STUDIES OF THE ANTICANCER EFFECTS OF THE VASCULAR TARGETING AGENT COMBRETASTATIN A-4 DISODIUM PHOSPHATE (CA4DP)

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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This dissertation is dedicated to my husband, Guoyi, and my parents for their unlimited love and support.

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Combretastatin A-4 disodium phosphate (CA4DP) is a tubulin-binding agent which has been shown to lead to rapid vascular shutdown in a variety of tumor models. The present studies were undertaken to gain insight into the mechanism(s) of action of CA4DP and to evaluate the antitumor efficacy of CA4DP either alone or in combination with conventional anticancer therapies in a xenograft model of Kaposi's Sarcoma (KS).

Initial studies that compared the responses of normal and neoplastic cells to CA4DP demonstrated that CA4DP had selective activity against proliferating endothelial cells. Further studies showed that CA4DP treatment resulted in a time dependent tubulin depolymerization in HMVEC-L cells. Tubulin disruption directly affected the ability of endothelial cell migration and attachment. Studies carried out

with a clonogenic cell survival assay demonstrated that CA4DP selectively reduced the number of viable proliferating HMVEC-L cells in a dose dependent manner. Moreover, CA4DP induced the death of proliferating endothelial cells predominantly by apoptosis. These findings provide a basis for the *in vivo* efficacy of CA4DP and its selective action against the proliferating endothelial cell population found in tumors.

In vivo studies using Hoechst-33342 staining demonstrated that a single 100 mg/kg dose of CA4DP caused a rapid vascular shutdown in KS xenografts. Histological evaluation showed evidence of morphological damage of tumor cells within a few hours after drug treatment, followed by extensive haemorrhagic necrosis. Studies using an *in vivo* to *in vitro* clonogenic cell survival assay further demonstrated that administering increasing doses of CA4DP to tumor-bearing mice resulted in a dose-dependent increase in tumor cell killing. CA4DP also significantly enhanced the antitumor effects of radiation and chemotherapeutic agents (cisplatin and vinblastine) in combination treatment studies. Moreover, repeated doses of CA4DP treatment either alone or in combination with cisplatin treatment caused significant growth delay in KS xenografts. These findings suggest that CA4DP ought to be considered as a candidate agent for therapeutic evaluation in patients with AIDS-KS in future Phase II clinical studies.

CHAPTER 1
INTRODUCTION

Cancer is characterized by progressive growth of cells that have lost their proliferative control. These cells ultimately destruct tissue and metastasize to organs distant from the primary site. In general, only one of three cancer victims can be cured by a single treatment modality, usually surgery, radio- or chemotherapy. The main problem with conventional cancer treatments, primarily chemotherapy and radiation therapy, is that they do not have high specificity for cancer cells. For radiation therapy, a degree of specificity is achieved by localizing the radiation to the tumor and its immediate surrounding normal tissue. For anticancer drugs, it is the rapid proliferation of the cancer cells that makes them more sensitive to cell killing than their normal cell counterparts. However, both modalities are limited by their toxicity to normal cells. In the case of radiotherapy, normal tissue surrounding the tumor limits the radiation dose, whereas for anticancer drugs, it is usually the killing of rapidly dividing normal cells, such as those in the bone marrow, hair follicles, and epithelial cells lining the gastrointestinal tract, that limit the dose that can be given. To achieve more tumor-specific treatment, differences between normal and malignant cells are being exploited. The physiology of solid tumors at the microenvironmental level provides a unique and selective target for cancer treatment.

The Unique Physiology and Microenvironment of Solid Tumors

Abnormal Tumor Vasculature

The physiology of solid tumor differs from that of normal tissues in a number of important aspects. A critical difference between tumors and normal tissues is the abnormal nature of the tumor microcirculation compared to the well-defined microvascular architecture of normal tissues (Konerding *et al.*, 1995). Solid malignant tumors are composed of both cancerous cells and normal host component. Tumor growth, resulting from uncontrolled neoplastic cell division, is absolutely dependent on a parallel proliferation of the nonmalignant cells which comprise the tumor vasculature. There are two types of vessels in tumor tissues: the existing vessels in normal tissues into which the tumor has invaded; and tumor microvessels arising from neovascularization resulting from increased expression of proangiogenic factors produced by tumor cells (Brown and Giaccia, 1998). Both types of vessels develop structural and physiological abnormalities that have become a hallmark of the tumor microvasculature. Studies have shown that tumor blood vessels are highly irregular, tortuous, have arterio-venous shunts, blind ends, lack smooth muscle or enervation, and have incomplete endothelial linings and basement membranes (Grunt *et al.*, 1985; Dewhirst *et al.*, 1989; Shah-Yukich and Nelson, 1988) (Figure 1-1). As a result, blood flow is often sluggish, highly irregular, and the vessels are much "leakier" than those in normal tissues. Tumor blood supply is therefore characterized by both spatial and temporal heterogeneity in both structure and function.

Hypoxia

In neoplastic tissue, there is a disproportionate relationship between tumor tissue and its vascular supply. Tumors are said to "outgrow" their blood supply; neovascularization lags behind the increase in the number of neoplastic cells (Tannock, 1970). As a consequence, the vascular network fails to provide adequate nutritional support and leads to heterogeneous tumor microregions varying in concentrations of oxygen, glucose, and other nutritional factors, as well as metabolic waste products, both within and among tumors of the same pathological grade and stage (Vaupel *et al.*, 1989; Vaupel *et al.*, 1996). In most solid malignancies the tissue O_2 status is poorer than in normal tissue at the site of tumor growth. For example, the medium pO_2 measured with electrodes for human breast tumors was 28 mm Hg, whereas that of normal breast was 68 mm Hg (Vaupel, 1994).

Due to the irregular blood flow and high interstitial pressure, some therapeutic agents are poorly delivered to tumors. Cells located distant from the functional blood supply, often hypoxic cells, could be resistant to drug therapy because of three factors: (a) they are exposed to lower concentrations of drug than those adjacent to blood vessels, primarily as a result of the metabolism of such agents through successive cellular layers; (b) as a result of a decline in nutrient and O_2 availability, cells further away from the vascular system would be dividing at a reduced rate (Amellem and Pettersen, 1991; Pallavicini *et al.*, 1979); an important consequence of this hypoxia-induced inhibition of proliferation is that because most anticancer drugs are primarily effective against rapidly dividing

cells, their effectiveness would be expected to fall off as a function of distance from blood vessels (Tomida and Tsuruo, 1999; Reynolds *et al.*, 1996); (c) oxygen-deficient cells may be inherently more resistant (Teicher, 1994).

Tumor hypoxia is also an important factor leading to resistance to radiotherapy. In many series of human tumors at different sites, roughly half the tumors have a median value of less than 10 mm Hg (Vaupel *et al.*, 1991; Nordsmark *et al.*, 1994). This median of 10 mm Hg is significant in that this is the point at which radiation resistance starts to develop with full resistance at values of less than 0.5 mm Hg (Brown, 1999). A typical radiation killing curve for mammalian cells under aerobic and hypoxic conditions is shown in Figure 1-2. The difference in radiation sensitivity between the aerobic and hypoxic cells, which is known as the oxygen enhancement ratio, is normally in the range 2.5-3 for mammalian cells. This effect, coupled with the finding that both human and rodent tumors possess regions of tissue oxygenation below 10 mm Hg, indicates why tumor hypoxia remains a key focus of research in radiobiology and radiotherapy.

Recent studies have shown that hypoxia in solid tumors has an important consequence in addition to conferring a direct resistance to radiation and chemotherapy. Tumor hypoxia also stimulates tumor progression by promoting angiogenesis through the induction of proangiogenic proteins such as vascular endothelial growth factor (VEGF) (Shweiki *et al.*, 1992). Clinical studies with soft tissue sarcomas (Brizel *et al.*, 1996) and with carcinoma of the cervix (Sundfor *et al.*, 1998) have shown that hypoxia is an independent and highly

significant prognostic factor predisposing tumors to metastatic spread. Therefore, tumor hypoxia also is seen as a predisposing factor toward increased malignancy and metastasis.

Because of their potential importance for treatment outcome, a large effort has been afforded over the years to identifying strategies that will reduce or eliminate hypoxic cells within solid tumors. A number of strategies to improving tumor oxygenation are being investigated, including high oxygen content gas breathing either alone (Fenton and Siemann, 1995) or coupled with the agent nicotinamide (Horsman *et al.*, 1994; Siemann *et al.*, 1994), right shifting of the oxyhemoglobin curve (Siemann and Macler, 1986; Hirst and Wood, 1987), and use of agents that increase tumor blood flow (Vaupel and Menke, 1989). A second approach is to use chemical sensitizers that mimic oxygen's ability to increase the sensitivity of hypoxic cells to radiotherapy and chemotherapy (Phillips and Wasserman, 1984). Over the past decade, several potent bioreductive cytotoxins, such as E09 and tirapazamine, agents whose cytotoxic activity is dramatically enhanced when they are metabolized in a hypoxic environment, have been identified (Workman, 1992; Brown and Siim, 1996). Specifically attacking the hypoxic cell subpopulations with bioreductive agents has a greater therapeutic potential than oxygenating the cells or chemically sensitizing them to radiation or chemotherapy. Not only is the killing tumor specific (hypoxic is tumor specific), but the cells killed are the ones resistant to conventional therapies. This principle of "complementary cytotoxicity" is illustrated in Figure 1-3. The combined killing of two agents with complementary

cytotoxicity is potentially much greater than that of two agents acting on the same cell population (Brown and Siim, 1996).

pH

Besides reduced oxygen level, pH is another microenvironmental characteristic which impacts on the therapeutic outcome of solid tumors. Tumors have been shown to have an acidic microenvironment compared to normal tissues (Wike-Hooley *et al.*, 1984). Increased capacity of glycolysis with the resultant production of lactic acid, lactate production via the breakdown of glutamine, and CO_2 production as a result of cellular respiration may contribute to the shift to acidic pH in malignant tissues.

The pH status of malignant tissue can also significantly influence drug activity, especially that of compounds which are weak acids and bases. Studies have demonstrated that selective decrease of the extracellular pH decreases the uptake and activity of weak bases such as vinblastine, but increases the uptake and cytotoxicity of weak acids such as chlorambucil (Gerweck and Seetharaman, 1996; Parkins *et al.*, 1996). Moreover, pH-induced alterations in drug stability, active transport processes, drug reactivity, activity of enzymes involved in localized drug activation and the interaction of the drug with its molecular target could all alter treatment efficacy.

Tumor Angiogenesis and Vasculature Targeting

Tumor Angiogenesis

Angiogenesis plays a significant role during normal growth, in physiological conditions (e.g., in the placenta and endometrium), and in pathological conditions

such as inflammation, wound healing, and tumor growth. Angiogenesis is thus not a specific phenomenon in tumors or a pathological condition, but instead an integral element of numerous different normal and pathological conditions.

Angiogenesis is a complex multistep process involving extracellular matrix remodelling, endothelial cell migration and proliferation, and capillary differentiation and anastomosis, which are regulated by angiogenic peptides (Blood and Zetter, 1990). The newly formed vessels are usually thin-walled capillaries or sinusoids with little more than an endothelial lining, backed by a basement membrane. Mobility and remodelling from pre-existing vasculature is an important component of angiogenesis. Many angiogenic factors that stimulate proliferation and migration of endothelial cells have been described (Pluda, 1997; Teicher, 1995). Amongst the most potent and specific factors for vascular growth is vascular endothelial cell growth factor (VEGF) (Claffey and Robinson, 1996; Zhang *et al.*, 1995). VEGF exists in several isoforms produced from one gene. It binds to VEGF receptors of which there are two, VEGFR1 (flt-1) and VEGFR2 (flk-1/KDR). It is generally thought that VEGFR2 is the most important for angiogenesis while VEGFR1 is expressed on macrophages and stimulates their migration (Jain *et al.*, 1996).

In normal adults, angiogenesis is limited to specific reproductive organs, and the growth and turnover of vascular endothelial cells in most tissues is measured in months and years (Hobson and Denekamp, 1984). Unlike what is found in most normal tissues, vessels in tumors contain populations of actively dividing endothelial cells in response to angiogenic factors. In human tumors the number

of dividing endothelial cells may be 50 times greater than in normal tissue (Harris, 1998). The vascularization of solid tumors is a prerequisite if a clinically relevant size is to be reached. Without sufficient vascular supply, no solid tumor can grow beyond a few cubic millimeters (Ausprunk and Folkman, 1977; Folkman, 1986). Further tumor growth depends on nutrient supply via a network of microvessels (Denekamp, 1993) which can be acquired, in part, by incorporation of existing host blood vessels. However, it is now well established that the majority of tumor blood vessels are newly formed as a result of angiogenesis triggered by the release of stimulators such as VEGF (Siemeister *et al.*, 1998). Thus, neovascularization is a critical aspect of a tumor's growth and development. Furthermore, angiogenesis is also essential for systemic metastasis, and recently it has been shown to be essential for local invasion (Skobe *et al.*, 1997).

Anti-angiogenesis Approach

The utter dependence of the tumor on its induced vessel formation for growth, survival and spread has created a great deal of enthusiasm for developing therapeutic approaches to specifically targeting the tumor vasculature (Folkman, 1995; Denekamp, 1993). A variety of approaches are under investigation. One approach, and most extensively studied, involves attempts to prevent the development of the vascular supply by inhibiting angiogenesis (Ingber *et al.*, 1990; O'Reilly *et al.*, 1994; Scott and Harris, 1994). Three options have been considered so far: (1) inhibition of the turnover from an avascular primary tumor into a fully vascularized tumor; (2) slowdown of tumor progression by preventing a tumor from becoming highly vascularized; (3) prevention of neovascularization

of distant metastases. Antiangiogenesis therapy targets a process that, under most circumstances, is tumor specific and therefore likely to have few normal tissue side effects.

The antiangiogenesis strategy includes agents which interfere with delivery or export of angiogenic stimuli (Schweigerer, 1995), antibodies to inhibit/inactivate angiogenic factors after their release (Mesiano *et al.,* 1998), antisense therapies (Im *et al.*, 1999), drugs which inhibit receptor action (Witte *et al.*, 1998), and inhibitors of endothelial cell proliferation (Boehm *et al.*, 1997). Several of the agents have moved forward to the clinic including metalloproteinase inhibitors, pentosan polysulphate and TNP-470 (Marshall and Hawkins, 1995; Denis and Verweij, 1997; Twardowski and Gradishar, 1997). Because of the major importance of VEGF as an angiogenic factor, numerous strategies are presently being used to inhibit VEGF activity in tumors. These have included antisense VEGF mRNA, monoclonal antibodies, and VEGF receptor inhibitors (Dvorak *et al.*, 1995; Kim *et al.*, 1993).

It is now recognized that angiogenesis is regulated by a balance between pro-angiogenic and anti-angiogenic factors and that loss of inhibitors may be an early stage in tumor progression. Angiogenesis inhibitors include thrombospondin (Taraboletti *et al.*, 1997), several cytokines (IL-4, IL-12) and proteolytic breakdown products of several proteins, including prolactin (Clapp and Delaescalera, 1997), plasminogen (Cao *et al.*, 1997) and collagen XVIII (O'Reilly *et al.*, 1994). It is these inhibitory peptides that have raised hopes that specific inhibition of tumor angiogenesis may be possible with minimal toxicity and high

efficiency. These angiogenesis inhibitors, particularly angiostatin and endostatin, are being actively investigated (O'Reilly *et al.*, 1994; Boehm *et al.*, 1997). They are specific inhibitors of endothelial cell proliferation and have no obvious effect on resting endothelial cells, nor on a variety of normal, transformed or neoplastic cells. Studies have shown that systemic administration of endostatin to tumor-bearing mice resulted in regression of tumors to a microscopic size (Boehm *et al.*, 1997). A dormant state, without evidence of toxicity, could be maintained for as long as endostatin was administered. Whether these very encouraging results will remain to hold true in the future, remains to be seen.

Vascular Targeting Approach

The concept of anti-angiogenic therapy relates to interfering with the stimulating substances that cause new vessel formation. This should be important in preventing establishment of small solid tumors or in preventing metastases. By contrast, another approach, the so-called vascular targeting approach, focuses on the use of agents that can directly destroy existing tumor vessels (Denekamp, 1990; Denekamp and Hill, 1991). Figure 1-4 illustrates the differences between anti-angiogenic and vascular targeting approaches. The functioning vascular network in tumors is pivotal for the survival of the tumor cells. This is confirmed by the fact that artificial induction of ischaemia by clamping off the tumor-feeding blood supply results in extensive tumor cell death and, if prolonged, tumor cures (Denekamp, 1993; Chaplin and Horsman, 1994). Such studies emphasize the therapeutic potential of strategies that target the tumor vasculature. The antivascular approach aims to cause a rapid shutdown in the vascular function

of the tumor, leading to extensive secondary tumor cell death. Since thousands of tumor cells are dependent on each tumor capillary for their metabolic requirements, an agent which induced even limited damage to these vessels could produce a cascade of tumor cell death (Denekamp, 1984; Denekamp, 1993).

Several features make tumor vasculature a suitable target in cancer therapy. While tumor cells are genetically unstable, rapidly mutating, and able to develop multidrug resistance, vascular endothelial cells are genetically stable and unlikely to become drug resistant (Folkman *et al.*, 1997). Drug delivery is not a problem, since the target cells directly line the blood stream. Perhaps most importantly, tumor vasculature represents an actively growing endothelium whereas the endothelium in most normal tissues is essentially dormant (Denekamp *et al.*, 1993). This last feature may provide a key difference between the tumor and normal tissue which can be exploited.

Development of antibodies to specific epitopes on the tumor vasculature (Burrows and Thorpe, 1993; Huang *et al.*, 1997) and vascular-targeted gene therapy (Chaplin and Dougherty, 1999) are two approaches that are receiving considerable attention. As mentioned previously, the advantages of targeting endothelium include that it reduces delivery problems and the number of cells that need to be targeted. These factors make the tumor vasculature a potentially ideal target for antibody- and gene therapy-based approaches. For antibody-based strategies, there is a need to identify antibodies that target unique determinants which are selectively and constitutively expressed on the tumor endothelium. Several studies are now underway to identify such antibodies. Potential

candidates include TEC-11, which recognized endoglin, and others that recognize the N-terminal domain of VEGF, $\alpha_v\beta_3$ integrin and the receptor tyrosine kinase Tie-1 (Brooks *et al.*, 1994; Burrows *et al.*, 1995). Gene therapy constitutes a potentially powerful means of selectively targeting the tumor-associated vascular endothelial cells, while at the same time minimizing the damage inflicted on various normal tissues. Candidate genes are expected to either directly kill vascular endothelial cells or sensitize them to the cytotoxic effects of ionizing radiation and/or chemotherapeutic agents. The most promising ones are genes encoding toxic protein inhibitors as well as genes that can convert relatively not-toxic prodrugs to their biologically active metabolites (Parentesis *et al.*, 1992; Deonarain *et al.*, 1995).

Besides antibody-based and gene therapy approaches, drug- and cytokine-based approaches to vascular targeting are also possible. Several agents that elicit irreversible vascular shutdown selectively within solid tumors have been identified. These include flavenoids such as flavone acetic acid (FAA) and more recently DMXAA (Zwi *et al.*, 1994), and tubulin binding agents such as colchicine and vinblastine. FAA was shown to have a broad spectrum of activity against solid tumors (Corbett *et al.*, 1986; Hill *et al.*, 1995). The action of FAA has been attributed in large part to its ability to induce the release of tumor necrosis factor-alpha (TNF-α) from tumors *in situ* (Cliffe *et al.*, 1994; Mahadevanv *et al.,* 1990). In contrast, the tubulin binding agent vinblastine causes little or no increase in plasma TNF-α levels in tumor-bearing mice (Hill *et al.*, 1995). Nevertheless, antivascular effects are a common feature of tubulin

binding agents. Chaplin *et al.* (1996) assessed the effects of vinblastine and four other tubulin binding agents (dolastatin 10, dolastatin 15, combretastatin A1 and combretastatin A4) on tumor blood flow. It was shown that all five agents induced a reduction in tumor blood flow range from 50% to 90%. The mechanisms governing these tumor-selective effects of tubulin binding agents are largely unknown, but it is possible that the inhibition of tubulin polymerization affects endothelial cell shape, leading to thrombus formation or changes in permeability of the endothelium.

Despite the reported antitumor effects, the clinical potential of vascular targeting strategies ultimately will be largely determined by the selective toxicity of the reagents. Unfortunately, to date, most of these agents have been reported to only elicit antivascular effects at doses approaching the maximum tolerated dose (MTD) and only in the presence of significant morbidity (Chaplin *et al.*, 1996). For example, vinblastine and colchicine markedly reduced tumor perfusion and caused necrosis of tumor tissue only when the injected dose was increased to lethal range (Nihei *et al.*, 1999). To fully appreciate the anti-vascular strategy, new agents with a large therapeutic window and improved selectivity are needed.

Combretastatin A-4 Disodium Phosphate

The tubulin and actin cytoskeleton are critical mediators for a number of important endothelial cellular functions other than the mitotic spindle and chromosome segregation. They facilitate intracellular organization, cell morphology, cell motility and the intracellular transport of molecules from the site of synthesis to the cell surface via microtubule motor proteins (Avile, 1992). The

cytoskeleton is a dynamic structure and conformational rearrangements occur in response to the endothelial cells' environment and exposure to mechanical forces (Cucina *et al.*, 1995). Because of the pivotal role the cytoskeleton plays with respect to cell shape in endothelial cells, it is not surprising they have effects on overall vascular function.

One class of tubulin-binding compounds which has received attention in recent years is the combretastatins. The African bush willow tree *Combretum Caffrum* is the source of 17 natural combretastatins and a further 22 similarly structured agents have been synthesized, thus making four series of compounds (named A to D) (Pettit *et al.*, 1987; O'Brien, 1997). Structurally, combretastatins consist of two substituted benzene rings linked by a saturated, hydroxy-substituted 2-carbon bridge (Figure 1-5). These combretastatins show structural similarity to colchicine and are competitive inhibitors of the binding of colchicine to tubulin (Pettit *et al.*, 1989; Sackett, 1993). They inhibit microtubule activity and interfere with cell growth and proliferation (Pettit *et al.*, 1989). The mechanism of their binding to tubulin was examined indirectly for one of them, combretastatin A-4, by evaluating their effects on the binding of radiolabeled colchicine to the protein (Lin *et al.*, 1989). Studies showed rapid binding of combretastatin A-4 to tubulin even at 0 degrees (binding was complete at the earliest times examined), in contrast to the relatively slow and temperature-dependent binding of colchicine. It demonstrated that the effectiveness of combretastatin A-4 as antimitotic agents appears to derive primarily from the rapidity of their binding to tubulin.

Combretastatin A-4 showed some concentration-dependent cytotoxicity against a variety of human tumors (El-Zayat *et al.*, 1993). As combretastatin A-4 itself is poorly soluble in water, a prodrug, combretastatin A-4 disodium phosphate (CA4DP) was prepared. This extra phosphate group can readily be cleaved by endogenous nonspecific phosphatases (O'Brien, 1997). Combretastatin A-4 binds to plasma protein, and this seems to reduce its activity (Tozer *et al.*, 1999). Therefore, there may be an advantage for using CA4DP rather than combretastatin A-4 beyond its increased solubility. Figure 1-5 shows the chemical structures of combretastatin A-4 and CA4DP. Both agents have been shown to lead to rapid vascular shutdown in several preclinical tumor models (Dark *et al.*, 1997; Li *et al.*, 1998; Horsman *et al.*, 1998). Recent studies continued to demonstrate the vascular effect of CA4DP. When assessed in a rat system, 100 mg/kg CA4DP caused a very large decrease in tumor blood flow, which by 6 hr, was reduced approximately 100-fold (Tozer *et al.*, 1999). Calculation of vascular resistance revealed some vascular changes in the heart and kidney for which there were no significant changes in blood flow. Using magnetic resonance imaging (MRI), Beauregard *et al.* (1998) showed that tumor perfusion decreased significantly in the central region of murine tumors after CA4DP treatment and it was consistent with the haemorrhage seen in histological sections. Further preclinical studies have demonstrated that avascular nodules do not appear to be responsive to this agent, providing additional evidence for a vascular mechanism of action (Grosios *et al.*, 1999). The rapid, selective and extensive

damage caused to the tumors by CA4DP has highlighted the potential of the agent as a novel cancer chemotherapeutic agent. More importantly, CA4DP typically produced these effects at concentrations less than one-tenth of the MTD thus offering a wide therapeutic window (Dark *et al.*, 1997; Li *et al.*, 1998; Horsman *et al.*, 1998).

Combretastatin is now in Phase 1 clinical trials in UK and US. The current studies are very encouraging (Randal, 2000). A patient with an anaplastic thyroid tumor, which was unaffected by standard therapies and progressed relentlessly, was treated with intravenous infusion of combretastatin. Three weeks after the first infusion, the tumor began to shrink. After six more infusions, CT and MRI failed to show any trace of the tumor. Later exploratory surgery confirmed the tumor's total disappearance. There also have been other patients whose previously very aggressive cancers – some of them metastatic – stabilized after treatment with combretastatin for 24 weeks or more (Randal, 2000).

In our laboratory, we have used several animal models to study the antitumor efficacy of CA4DP. Treatment with CA4DP has been shown to produce extensive regions of hemorrhagic necrosis in both murine tumors (Li *et al.*, 1998) and human tumor xenografts. Moreover, as the application of antivascular strategies will need to be given in conjunction with conventional anticancer therapies, we also examined the efficacy of combining CA4DP with ionizing radiation. Such treatment may be required to destroy the remaining rim of tumor cells surviving at the periphery near normal tissue vessels. Results from our laboratory indicate

superior antitumor efficacy in the combination of CA4DP and radiation (Figures 1-6 and 1-7).

Significance

It has been well established that the vascularization of solid tumors is a prerequisite if a clinically relevant size is to be reached. The dependence of the tumor on its induced vessel formation has created a great deal of enthusiasm in specifically targeting the microcirculation in cancer therapy. The central goal of this project was to investigate the potential therapeutic utility of vascular targeting drug CA4DP. Experiments proposed in this project were directed at gaining a better understanding of the mechanism of its action on tumor and endothelial cells, and to assess its efficacy in human tumor models either alone or in combination with conventional anticancer therapies. The thesis focused on a model of Kaposi's Sarcoma (KS), since AIDS-KS is a fulminant disease that usually requires aggressive treatment, especially when it involves visceral organs, which lacks effective therapies. Irradiation, systemic chemotherapy, and interferon (IFN-α), though helpful, are administered primarily for symptomatic relief and to prevent disease progression. Conventional treatments do not prolong survival, and their clinical effectiveness is not satisfying (Sung *et al.*, 1997). Continued pursuit of more effective agents clearly is needed. Given the possible future clinical impact of CA4DP, we believe it to be highly worthwhile to investigate effects of this agent in a preclinical model of KS.

The specific aims of this project are as follows:

Specific aim 1. To study the different responses of normal and neoplastic cells to CA4DP by examining the effects of CA4DP on cell cycle, cell survival, and apoptosis.

Specific aim 2. To gain further insight into the mechanisms underlying the antivascular action of CA4DP by examining the effects of CA4DP on human microvascular endothelial cells (HMVEC-L).

Specific aim 3. To examine the antitumor efficacy of CA4DP by applying histological approaches and by measuring its cytotoxic action in KS xenografts.

Specific aim 4. To assess the potential *in situ* therapeutic benefit of combining CA4DP with conventional anticancer therapies by evaluating the effects of such treatments in KS xenografts.

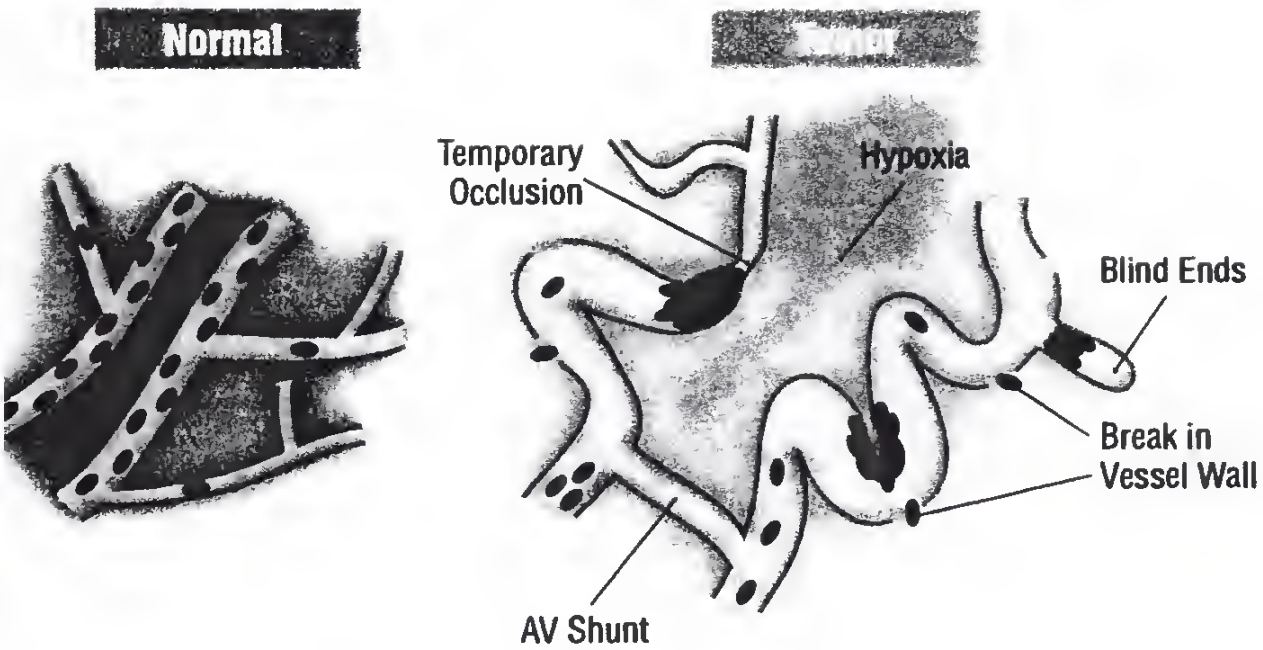


Figure 1-1. Diagram showing the principal differences between the vasculature of normal and malignant tissue. Whereas normal tissues have relatively uniform and well-ordered blood vessels that are sufficiently close together to oxygenate all of the tissue, blood vessels in tumors are tortuous, have imcomplete vessel walls, have sluggish and irregular blood flow, and have regions of hypoxia between the vessels (Brown and Giaccia, 1998).

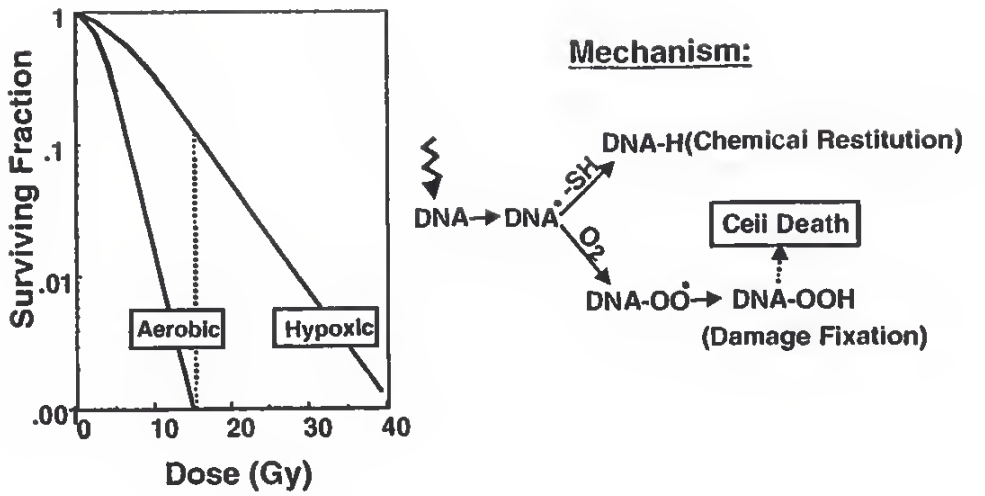


Figure 1-2. Typical survival curves to ionizing radiation for mammlian cells under aerobic and hypoxic conditions. Most mammalian cells, irrespective of genetic background, exhibit a survival curve with an Initial "shoulder" region followed by exponential cell killing. The oxygen enhancement ratio is typically 2.5-3.0. The dotted vertical line at 14 Gy shows the >2 logs difference in cell kill for aerobic and hypoxic cells at this dose. Also shown is the mechanism for the greater sensitivity of aerobic cells as compared to hypoxic cells, ionizing radiation produces a radical in DNA, which can be either chemically restituted by donation of hydrogen from nonprotein sulfhydryle (-SH) in the cell or, in the presence of oxygen, converted into permanent damage that increases the probability of cell death (Brown, 1999).

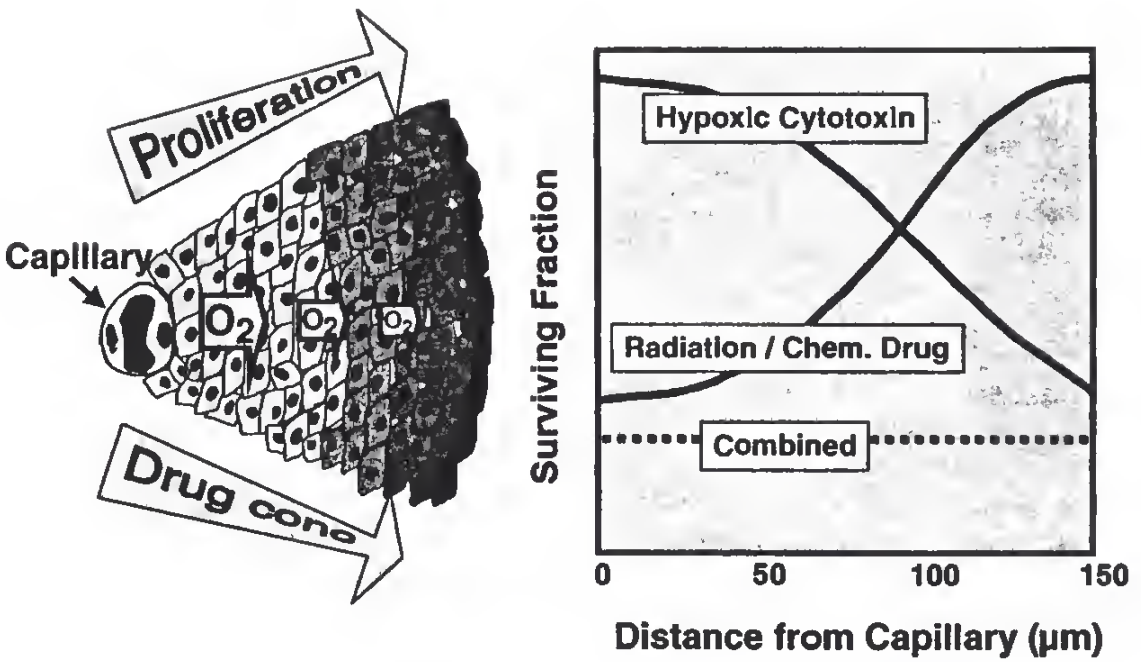


Figure 1-3. *Left*, a diagrammatical representation of part of a tumor cord surrounding a capillary showing decreasing oxygen concentration as well as decreasing cellular proliferation and drug concentration as a function of distance from the capillary. *Right*, the considerations on the left lead to the prediction that cell killing by radiation or most anticancer drugs will be reduced as a function of distance from the capillary. The combination of standard treatment with a hypoxic cytotoxin would be expected to overcome the problem of hypoxic cells by producing a relatively uniform cell profile of cell killing as a function of distance from the capillary (Brown and Siim, 1996).

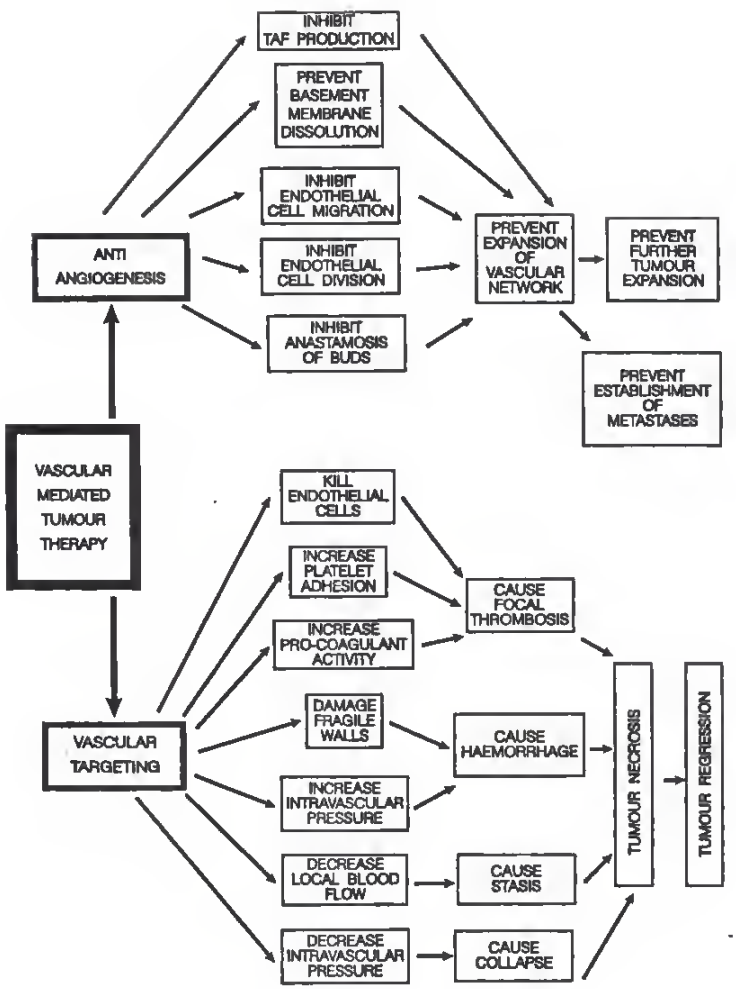
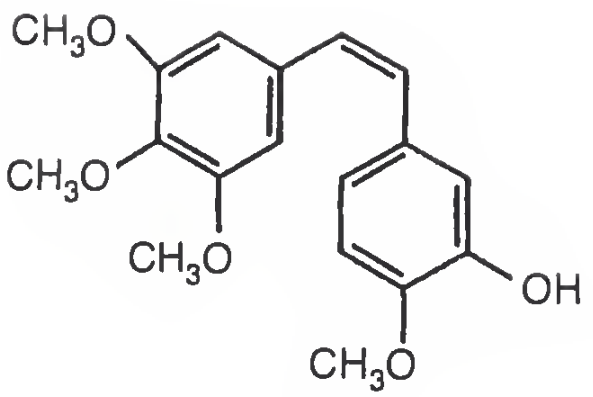


Figure 1-4. Diagram to illustrate the differences between the concepts and the likely outcome of anti-angiogenic strategies and those designed to produce ischaemic or haemorrhagic necrosis by vascular targeting (Denekamp, 1993).

a.



b.

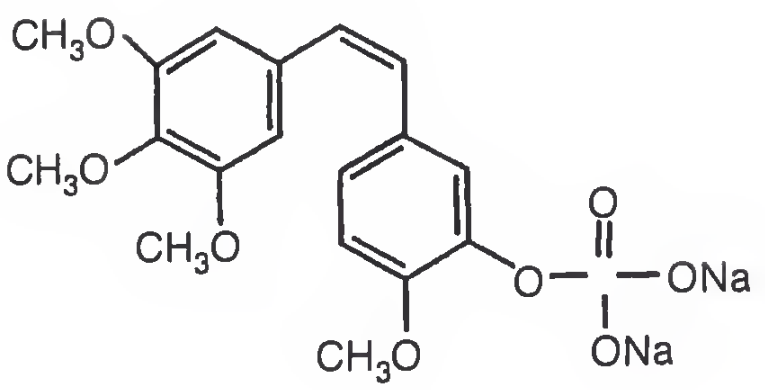


Figure 1-5. The chemical structures of combretastatin A-4 (a) and combretastatin A-4 disodium phosphate (CA4DP) (b)

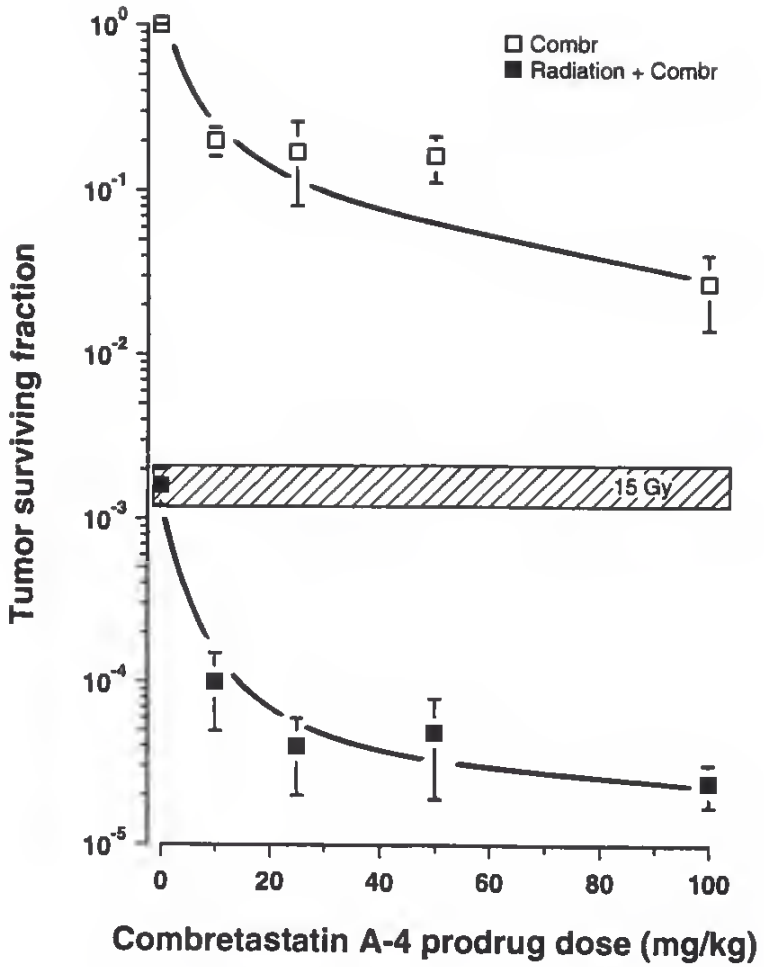


Figure 1-6. Tumor cell killing in KHT sarcomas treated with increasing doses of CA4DP either alone of 1 hr after irradiating the tumors with a 15-Gy dose of radiation. Data are the mean ± SE of 6-12 tumors.

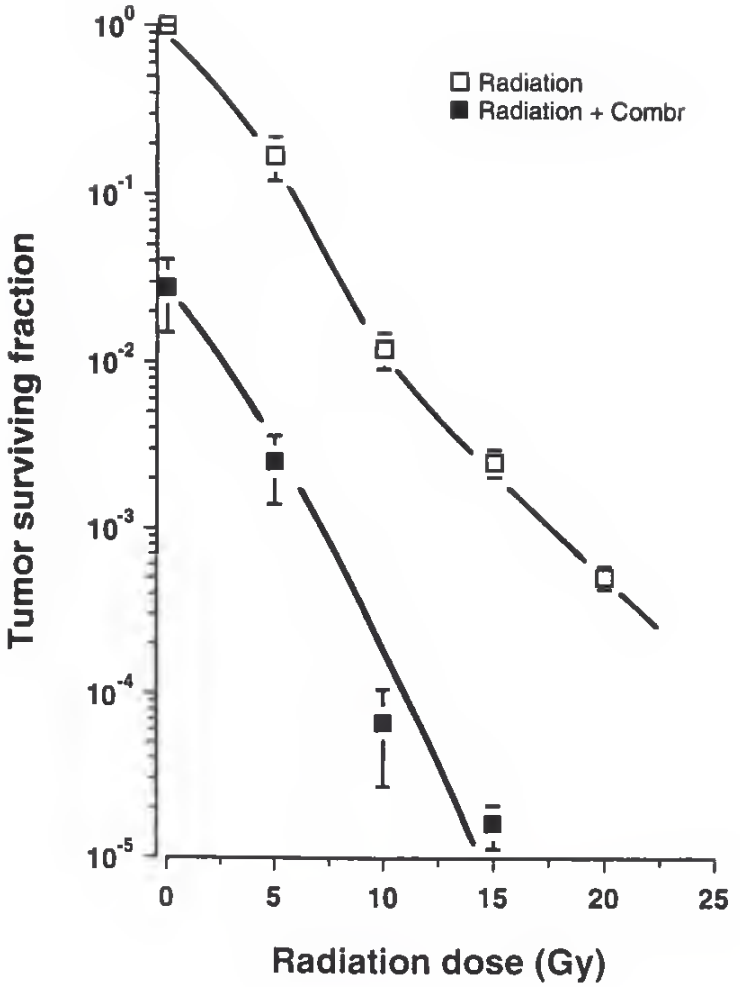


Figure 1-7. Tumor cell survival in KHT sarcomas treated with a 100 mg/kg dose of CA4DP 1 hr after a range of doses of radiation. Results are the mean ± SE of 3 experiments.

CHAPTER 2
COMPARISON OF THE DIFFERENT RESPONSES OF NEOPLASTIC AND NORMAL CELLS TO CA4DP *IN VITRO*

Introduction

The survival and growth of solid tumor deposits depends critically on the development of a blood vessel network. The functioning vascular network in tumors provides the tumor cells with oxygen and nutrients, and enables removal of the toxic waste products of cellular metabolism. The fact that the production of several angiogenic growth factors can be up regulated by physiological parameters, including low oxygen or glucose and acidic pH, which are associated with vascular insufficiency, provides a logical rationale for the strong angiogenic stimulus in malignant tissue (Chiarotto and Hill, 1999; Namiki *et al.*, 1995). The continued proliferation of tumor cells will result in deprivation of oxygen and glucose and production of acidic metabolites, thus stimulating the development of additional neovasculature (Siemeister *et al.*, 1998). The new vessels facilitate the further expansion of the tumor cell mass providing a perpetual loop. Clearly, the cycle can be interrupted by killing or inhibiting the growth of the tumor cells. However, interventions that compromise the function or growth of the tumor neovasculature can also be effective. Therefore, tumor blood vessels represent a central target for the development of new approaches to cancer therapy.

The majority of research work in this area has been focused on preventing the growth of new tumor vessels, so called anti-angiogenesis. Many agents that were identified as anti-angiogenic target at least one of the several stages involved in new vessel formation, i.e. basement membrane degradation, endothelial cell migration, endothelial cell proliferation and tube formation (Fan *et al.*, 1995). In contrast to the focus on anti-angiogenic approaches to therapy, there has, until recently, been relatively little effort afforded to the identification and development of therapies that specifically compromise the function of the existing neovasculature in solid tumors.

Interestingly, evidence for the therapeutic potential of vascular targeting approaches existed a lot earlier than recent studies. It had been reported over 150 years ago that occasionally solid tumors in the clinic could be eradicated when their circulation was interrupted either by torsion of the vascular pedicle or by thrombosis of a major feeding vessel (Walsh, 1844). The pivotal role of tumor vasculature and the effects of its selective destruction were also highlighted by Woglum over 75 years ago (Woglum, 1923). However, it is only within the last decade that research has been focused seriously on the development of therapies that specifically target and damage tumor neovasculature. Despite this limited development time, many promising approaches, including drug-, antibody- and gene therapy-based strategies have emerged.

Many tubulin-binding agents have been shown to have antivascular effects. The vinca alkaloids, for example vincristine and vinblastine, could induce vascular damage at doses close to the MTD (Baguley *et al.*, 1991; Hill *et al.*,

1995). However, they also have shown direct cytotoxic effects against a variety of tumor cells (Zhou and Rahmani, 1992). Although several studies have shown the antivascular effects of CA4DP in several clinical models (Dark *et al.*, 1997; Li *et al.*, 1998; Horsman *et al.*, 1998), it is not clear whether the activity of CA4DP is selectively against vascular endothelial cells or whether it also acts on tumor cells. In order to shed light on this, in the present studies, we examined the responses of human tumor cells (KSY-1 and A549), human endothelial cells (HMVEC-L), and human fibroblasts (FG1522) to CA4DP *in vitro*.

The tubulin and actin cytoskeleton are critical mediators for mitotic spindle formation and chromosome segregation. Because CA4DP is a tubulin-binding agent, studies were initiated by examining its effect on cell cycle distribution in different cell types. Later we examined the cytotoxicity of CA4DP and whether it could induce apoptosis in both neoplastic and normal cells.

Material and Methods

Cell Culture

Two human tumor cell lines, Kaposi's Sarcoma cell line KSY-1 (ATCC, Rockville, MD) and lung cancer cell line A549 (ATCC, Rockville, MD) were used in the study. KSY-1 cells were cultured in positively charged Cell$^+$ tissue culture flasks from SARSTEDT (Newton, NC). Cell$^+$ TC flasks provide a positively charged surface for difficult-to-grow adherent cell cultures. Both KSY-1 and A549 cells were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY) with 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum (FBS).

Human microvascular endothelial cells of the lung (HMVEC-L), obtained from Clonetics (San Diego, CA), were grown in EGM-2-MV medium (Clonetics) containing 5% FBS and supplements (0.1% hEGF, 0.4% hFGF-B, 0.1% VEGF, 0.1% Ascrobic Acid, 0.04% Hydrocortisone, 0.1% Long R3-IGF-1, 0.1% Heparin and 0.1% GA-1000). Clonetics Trypsin and Trypsin Neutralization Solution (TNS) were used for subculture.

Human skin fibroblasts FG1522 (from Dr. Hei's lab, Columbia University) were grown in Dulbecco's MEM Medium with 10% FBS and 25 μg/ml dose of gentamycin.

Drug Preparation

CA4DP (Oxigene Inc., Lund, Sweden) was dissolved in 5% sodium carbonate at a concentration of 10 mM and then subsequently diluted in 0.9% saline and culture medium immediately before use.

Cell Cycle Studies

A549, KSY-1, HMVEC-L, and FG1522 cells were plated in 60 mm dishes at 2×10^5 cells/dish. On day 3, the cells were exposed to various concentrations of CA4DP for a period of 2 hr. The dishes were then washed with PBS and replenished with fresh media. 22 hr later, the cells were trypsinized, counted, and fixed in 50% ethanol overnight. Before analyzed by FACS, the cells were treated with 1 mg/ml RNase (in PBS) for 30 min. The samples were then washed with PBS twice and resuspended in 25 mg/ml propidium iodide (PI) (in PBS) at a volume of 1×10^6 cells/ml. The cells were stained with PI in darkness for at least 10 min and were then analyzed by FACS for cell cycle distribution on a Becton

Dickinson flow cytometer made available through the University Core Facility for Flow Cytometry at the University of Florida.

Cell Viability Studies

Cell viability was determined using clonogenic cell survival assay. Briefly, A549, KSY-1, HMVEC-L, and FG1522 cells were plated in 60 mm petri dishes at 1×10^5 cells/dish. On day 3, the cells were exposed to various concentrations of CA4DP for a period of 2 hr or 24 hr. The dishes were then washed with PBS twice and the cells were trypsinized and counted. The cells were then mixed with 10^4 lethally irradiated cells and plated into 60 mm petri dishes. After 2 weeks of incubation at 37°C, colonies of 50 or more cells were counted with the aid of a dissecting microscope. Cell surviving fractions were calculated as the ratio of colonies counted in treated versus untreated group. To assess the effect against quiescent cells, the cells were first grown in 60 mm petri dishes and once confluent were treated and assayed as described above.

Apoptosis

Four cell lines each grown in 2-well chamber slides were treated with 50 μM CA4DP for 2 hr. The treated cells were fixed in 4% formaldehyde solution immediately after treatment or 22 hr later for TdT-mediated dUTP Nick-End labeling (TUNEL) assay. Basically, the cells were permeabilized in 0.2% Triton X-100 solution for 5 min. DNA strand breaks were then labeled with fluorescein-12-dUTP in TdT incubation buffer at 37°C for 1 hr. The samples were then counterstained with 1 μg/ml diamidine-2-phenylindole (DAPI), which binds to the A-T-rich regions of DNA. Localized green fluorescence of apoptotic cells

(fluorescein-12-dUTP) in a blue background (DAPI) was detected by fluorescence microscopy. The percentage of apoptotic cell was obtained by dividing the number of cells with green fluorescence by the total number of cells with blue fluorescence. A minimum of 300 cells were counted for each condition.

Results

To evaluate the effect of CA4DP on cell cycle distribution, proliferating normal and neoplastic cells were exposed to a range of doses of CA4DP for 2hr, and DNA profiles were analyzed by FACS 22 hr later. Results showed that CA4DP could effectively block all cell types in the G_2/M phase beginning at the doses ~0.05-0.1 μM (Figures 2-1 and 2-2). Increasing the drug dose to higher concentrations caused either a slight or no increase in the percentage of cells in the G_2/M phase in different cell types (Figure 2-2). It was also observed that, even at high concentrations, CA4DP arrested only 30-40% HMVEC-L and FG1522 cells in the G_2/M phase, whereas in A549 and KSY-1 cells there were almost complete G_2/M blocks.

Clonogenic cell survival assay was used to evaluate cell viability in each cell line treated with a range of doses of CA4DP. A 2-hr treatment, administered to exponentially growing cell populations, was found to be ineffective against FG1522 and A549 cells and showed only slight killing of KSY-1 cells (Figure 2-3; a-c). In contrast, under those conditions, CA4DP displayed significant dose-dependent activity against proliferating HMVEC-L (Figure 2-3d). Importantly this effect was specific to proliferating endothelial cells: quiescent HMVEC-L cells showed no response to CA4DP even at high doses (Figure 2-3d). With

prolonged drug exposure (24hr), CA4DP caused a clear dose-dependent cell kill in both A549 and KSY-1 cells, but still showed no toxicity to FG1522 cells. (Given the exquisite sensitivity of proliferating HMVEC-L to CA4DP, extended drug exposure times were not evaluated in this cell type.)

To gain further insights into the cytotoxic mechanisms of CA4DP, we also used TUNEL assay to examine the induction of apoptosis in the different cell types treated with this agent (Figure 2-4). The results showed that 2-hr treatment with 50 μM CA4DP assessed immediately or 22 hr later led to very little apoptosis in fibroblasts compared to the control group (0.6 and 1.2%, respectively). A549 cells showed a similar response; there was no induction of apoptosis immediately after CA4DP treatment, and 22 hr later, 4.6% of cells were detected as apoptotic cells. CA4DP induced more apoptosis in KSY-1 cells than in A549 cells. In this cell line the 0.7% apoptotic index in untreated KSY-1 cells rose to 2.8% immediately after treatment and to 7.9% 22 hr later. The results also showed that CA4DP induced the highest level of apoptosis in HMVEC-L cells. Immediately after a 2-hr CA4DP exposure 7.5% of cells underwent apoptosis. This number increased to almost 20% 22 hr later.

Discussion

The main aim of this study was to investigate whether CA4DP demonstrated any cell type specificity/selectivity by comparing its effects in normal and neoplastic cell types. Initial studies with FACS analysis showed that non-toxic concentrations of CA4DP caused arrests of the cells in the G_2/M phase in all the cells examined, indicating a disruption of mitosis due to a functional deficiency of

the tubulin apparatus, which disables the cells to divide their chromosomes properly. More importantly, the doses for CA4DP to initiate the cell cycle effect in different cell types were found to be within the same range (~0.05-0.1 μM). This suggested that CA4DP could bind to the tubulin in different types of cells with the same efficacy, thus resulting in cellular tubulin disruption and similar G_2-arrest effects at the same drug concentration. It was also observed that while CA4DP could cause almost a complete G_2-arrest in A549 and KSY-1 cells, only 30-40% of HMVEC-L and FG1522 cells were blocked in the G_2/M phase even at high CA4DP concentrations. We believe that this arises because unlike the tumor cell lines where the majority of cells are actively proliferating, a significant proportion of the normal cells may never enter the cell cycle. To attempt to examine this possibility we are currently utilizing the antibody against Ki67, a nuclear antigen for proliferating cells, and BUdR labeling to determine the fractions of proliferating cells in these cell populations.

Although CA4DP exerted similar effectiveness in terms of cell cycle arrest in both neoplastic and normal cells, results from the clonogenic cell survival assay showed significantly difference in the responses of the different cell types to this agent. CA4DP showed dose-dependent activity against proliferating endothelial cells with short drug exposure (2 hr). For example, a 20 μM drug concentration reduced viable HMVEC-L cells to less than 10% compared to the untreated cells. If the endothelial cells were quiescent during the drug exposure, no significant drug toxicity was observed. In contrast to the endothelial cells, 2-hr exposure to CA4DP caused no change in cell viability in proliferating FG1522 and A549

cells, and only a slight toxic effect in KSY-1 cells. These observations implied a selective toxicity of CA4DP toward proliferating endothelial cells. With prolonged drug exposure (24 hr), CA4DP did show toxicity against tumor cells (both A549 and KSY-1 cells). But over the dose range evaluated FG1522 cells were still not affected. We believe that the specific killing of dividing endothelial cells observed after CA4DP treatment might be critical for the *in vivo* action of this drug. Parenthetically the observation that normal cells may be less susceptible to CA4DP treatment may explain the low level of toxicity of CA4DP observed in the preclinical studies (Dark *et al.*, 1997).

Apoptosis is an active and gene-directed mode of cell death, involved in embryological development, organ involution, and the response of both normal and transformed cells to cytotoxic agents (Gorczyca *et al.*, 1993). It is characterized by rapid nuclear and cytoplasmic condensation and cellular disintegration into apoptotic bodies (Kerr *et al.*, 1972). Recent work has shown that apoptosis is controlled by a complex network of positive and negative signals, which originate either from specific gene products or from the extracellular environment (Stewart, 1994). The present studies showed that 50 μM CA4DP induced the highest level of apoptosis in endothelial cells compared to tumor cells and fibroblasts, demonstrating again the selective toxicity of CA4DP against endothelial cells.

It was observed that CA4DP caused cell cycle arrest in HMVEC-L starting at very low dose (0.05 μM). However, CA4DP started to show toxicity against HMVEC-L only at doses higher than 1 μM. The different effects of CA4DP

observed at low and high doses is because at low doses, CA4DP-induced G_2-arrest in the endothelial cells was reversible. Arrested cells recovered at 48-72 hr after 2-hr CA4DP treatment (data not shown). Only at higher doses ($\geq 1\mu M$), CA4DP caused irreversible G_2-arrest in HMVEC-L, thus leading to subsequent clonogenic cell death and cell apoptosis.

Taken together, the present results demonstrated that CA4DP displayed a significantly higher cytotoxicity toward proliferating endothelial cells than to tumor cells and normal human fibroblasts. This may well explain the selective antivascular effects of CA4DP observed in preclinical studies *in vivo*. Still the fundamental question of "Why CA4DP has such selectivity toward endothelial cells?" remains largely unknown. Nevertheless the selectivity of the effects are highly encouraging and clearly warrant more detailed study of the cellular and molecular mechanisms involved as well as the continued investigation of the therapeutic potential of the drug as an antivascular agent.

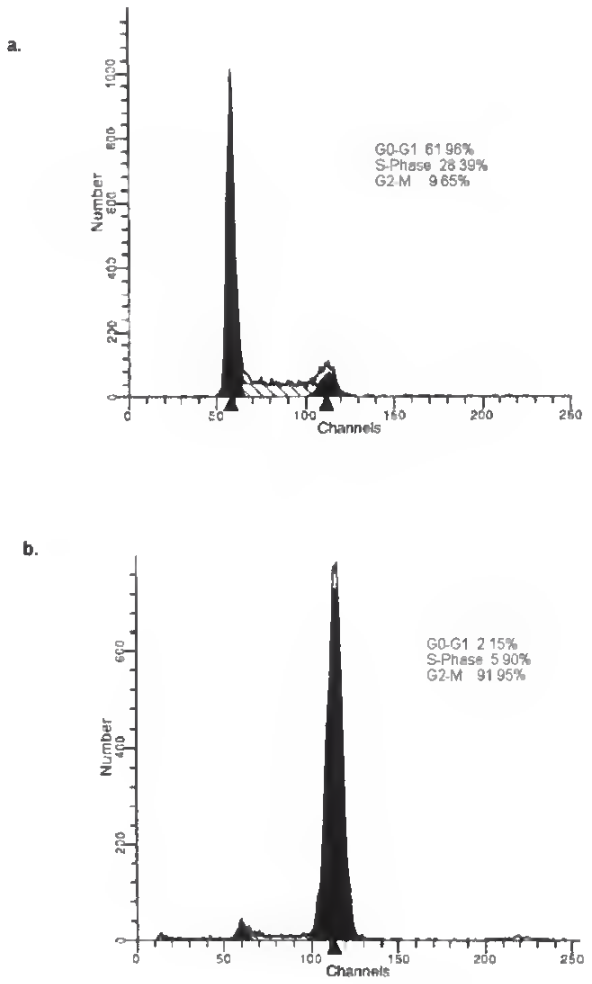


Figure 2-1. Analysis of cells cycle distributions in A549 cells by FACS. a) Untreated A549 cells; b) A549 cells treated with 0.1 μM CA4DP for 2 hr and fixed for FACS analysis 22 hr later.

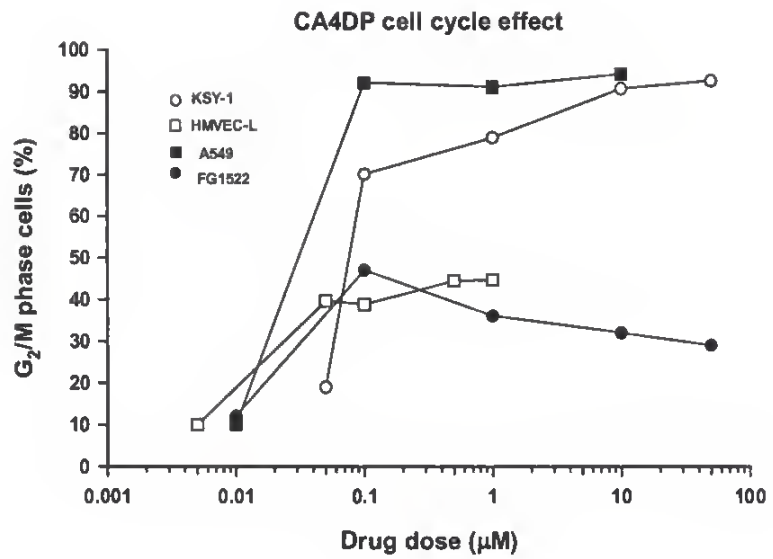


Figure 2-2. G_2-arrest caused by CA4DP treatment in FG1522, A549, KSY-1, and HMVEC-L cells. The cells were treated with a range of doses of CA4DP for 2 hr and fixed for FACS analysis 22 hr later.

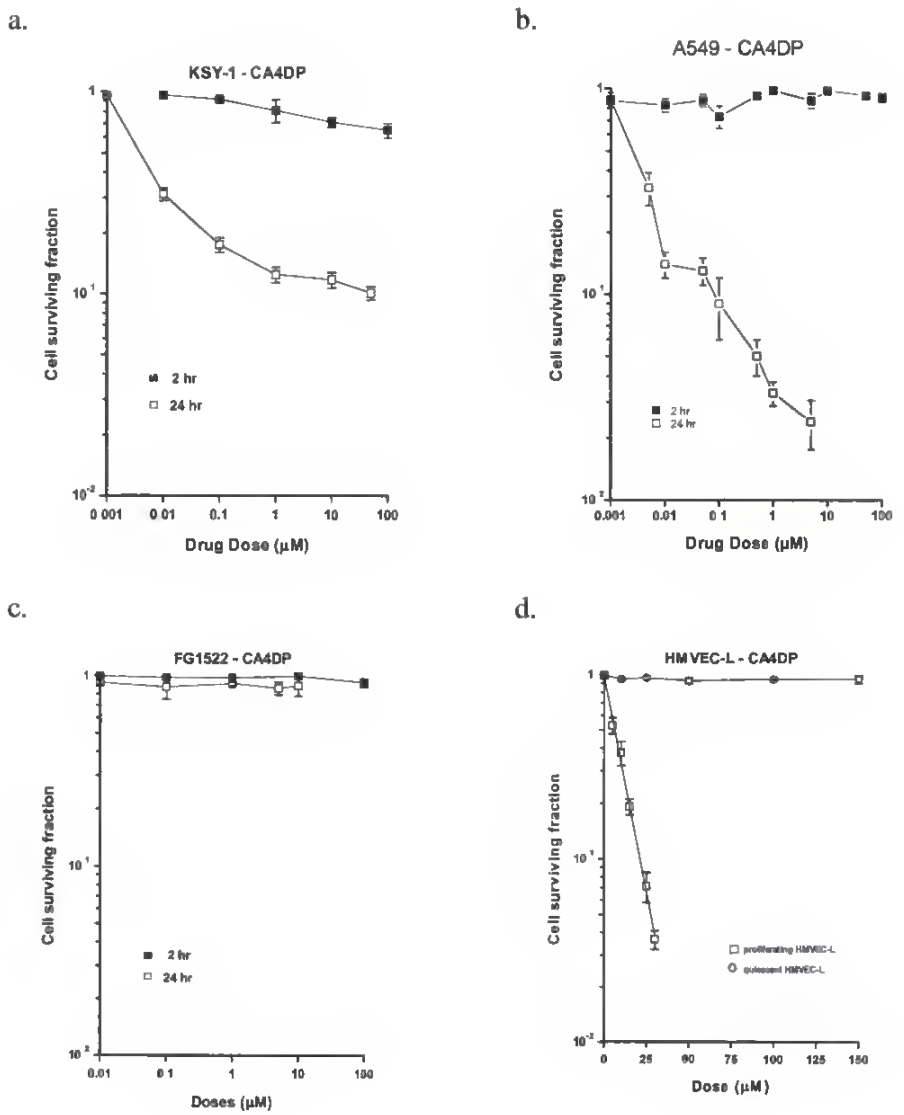


Figure 2-3. The cytotoxicity profile of CA4DP against KSY-1 (a), A549 (b), FG1522 (c), and HMVEC-L cells (d). The cells were exposed to CA4DP for 2 hr (a-d) or 24 hr (a-c) and the cell killing effects were assessed using clonogenic cell survival assays. Data are the mean ± SE of three experiments.

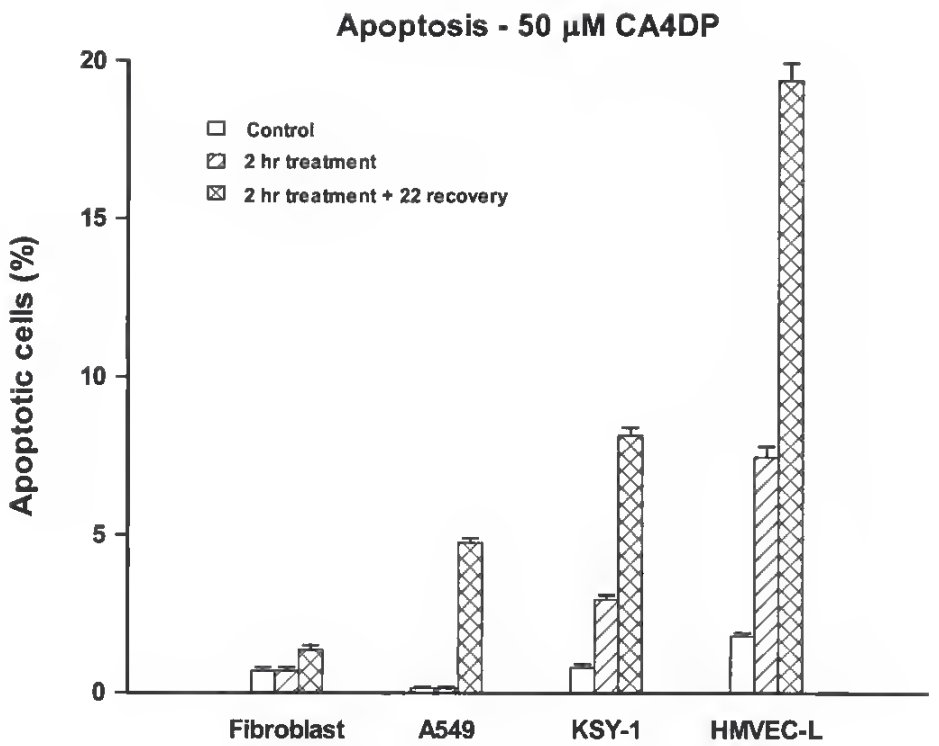


Figure 2-4. Effect of CA4DP on cell apoptosis in fibroblasts FG1522, A549, KSY-1, and HMVEC-L cells. The cells were treated with 50 µM CA4DP for 2 hr and stained for TUNEL assay either immediately after treatment or 22 hr later. Data are the mean ± SE of three independent experiments.

CHAPTER 3
EFFECTS OF CA4DP ON HUMAN MICROVASCULAR ENDOTHELIAL CELLS *IN VITRO*

Introduction

More than 10^{12} endothelial cells line the inside of blood vessels, covering a surface area of more than 1000 m^2 (Jaffe, 1987). Not only do they form the structural basis of blood vessels and provide an antithrombogenic surface, but they also contribute to numerous metabolic functions including coagulation and thrombolysis, control of vasotonus and antigen presentation, as well as basement membrane and growth factor synthesis (Pearson, 1991). Endothelial cells in the adult form a highly heterogenous cell population that varies in different organs. These cells normally are quiescent. After induction of angiogenesis by angiogenic cytokines, however, endothelial cells can proliferate as rapidly as bone marrow cells, which have a turnover time of 5 days (Folkman, 1995). Angiogenesis is a mode of endothelial cell activation that induces distinct phenotypic changes. Triggered by paracrine and autocrine mechanisms, it enables endothelial cells to break away from preexisting vessels to enter a complex morphogenetic cascade that will ultimately lead to the formation of new vessels with mature endothelial cells. Endothelial cells released in culture from growth arrest and allowed to migrate change their adhesive properties, their surface glycosylation pattern, their cytokine production, and their growth factor receptor expression pattern, as well

as their proteolytic balance (Augustin-Voss *et al.*, 1992; Weich *et al.*, 1991). Consequently, since angiogenesis is a developmentally regulated process that is down-regulated in the healthy adult (except for the female reproductive system), inhibition of angiogenic-specific cell functions might be useful for targeting new vessels during tumor growth.

One key in the development of treatment strategies is to identify differences that exist between the tumor vasculature and normal tissues. As mentioned before, the blood vessels in tumors are proliferating more rapidly than those in normal tissues (Denekamp, 1990). Thus, targeting features of proliferating endothelium, or even newly formed vasculature could achieve some selectivity. Another well-established feature of tumor blood vessels is that, unlike those in normal tissues, they can be subjected to low oxygen tension (Chaplin *et al.*, 1987; Hill *et al.*, 1996). A third obvious feature is that tumor endothelium is located adjacent to the malignant tumor cells, which in turn can alter endothelial cell characteristics. Exploiting the changes that such microenvironmental stimuli induce both in endothelial cell function and gene expression will undoubtedly provide the key to achieving effective and highly selective approach to targeting endothelial cells in tumors.

Vascular targeting approach aims to destroy the tumor vessels which contain rapidly proliferating endothelial cells. Antivascular effect is a common feature of tubulin binding agents. The original interest in the vascular-damaging effects of such agents was stimulated by studies with colchicine reported in the 1930s and 1940s. Studies clearly demonstrated that colchicine preferentially damaged newly

formed capillaries in tumors with the consequence of inducing hemorrhage and extensive necrosis (Ludford, 1945). Activity was noted in many different experimental tumor systems, but significant effects were only achieved at doses approaching the MTD. Later studies indicate that other tubulin binding agents, such as vincristine and vinblastine, at doses approximating the MTD could also induce vascular damage (Baguley *et al.*, 1991; Hill *et al.*, 1995). Recent studies focused on evaluating a number of inhibitors of tubulin polymerization for their ability to induce vascular damage in tumors (Chaplin *et al.*, 1996), aiming to identify agents with a superior therapeutic index for their vascular effects. Fortunately, combretastatin A-4 and CA4DP have been identified as agents that can induce vascular damage in tumors at doses much less than the MTD (Dark *et al.*, 1997; Beauregard *et al.*, 1998).

As with all approaches to cancer therapy, vascular targeting is only realistic if significant selectivity between tumor and normal tissue response can be achieved. *In vitro* studies from Chapter 2 revealed marked cytotoxic effects of CA4DP against proliferating but not quiescent endothelial cells. A549, KSY-1, and FG1522 cells were much less affected by CA4DP than the HMVEC-L cells. Why proliferating endothelial cells show such sensitivity to the *in vitro* effects of CA4DP is not known yet. Although the clinical potential of CA4DP has been recognized by its recent Phase I clinical studies in UK and US, further information is required regarding its mechanism of action. In light of the potent antivascular effects of CA4DP the present studies were undertaken to gain insight into the mechanism(s) of action of CA4DP by studying its activity in HMVEC-L.

Material and Methods

Cell Culture

HMVEC-L cells, obtained from Clonetics (San Diego, CA), were grown in EGM-2-MV medium (Clonetics) containing 5% FBS and supplements (0.1% hEGF, 0.4% hFGF-B, 0.1% VEGF, 0.1% Ascrobic Acid, 0.04% Hydrocortisone, 0.1% Long R3-IGF-1, 0.1% Heparin and 0.1% GA-1000). Clonetics Trypsin and Trypsin Neutralization Solution (TNS) were used for subculture.

Drug Preparation

CA4DP (Oxigene Inc., Lund, Sweden) was dissolved in 5% sodium carbonate at a concentration of 10 mM and then subsequently diluted in 0.9% saline and culture medium immediately before use.

Indirect Immunofluorescence

The intracellular distribution of microtubules following drug treatment was determined using indirect immunofluorescent staining (Giannakakou *et al.*, 1998; Woods *et al.*, 1995). HMVEC-L cells were plated in 35 mm dishes 1 day prior to treatment with CA4DP. Both treated and untreated cells then were fixed in 1:1 methanol/acetone for 5 min at room temperature and washed with PBS. Incubation with the primary anti-β-tubulin MAb for 70 min was followed by a 50-min incubation with the secondary flourescein-conjugated goat anti-mouse IgG antibody. All antibody incubations and washes were performed at room temperature. Morphological analysis then was performed by fluorescence microscopy.

Tubulin Polymerization Assay

HMVEC-L cells grown in 24-well plates were treated with 5 μM CA4DP for a specified time. After washing each well twice with 1 ml PBS (Ca^{2+} free), the cells were lysed at 37°C for 5 min in the dark with 100 μl of hypotonic buffer (20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% Nonidet P-40, 2 mM PMSF, 200 U/ml Approtinin, 100 μg/ml soybean trypsin inhibitor, 5mM ε-amino caproic acid and 1 mM benzamidine) (Giannakakou *et al.*, 1998). The wells were scraped and the lysates transferred to 1.5 ml Eppendorf tubes. Each well was rinsed with an additional 100 μl of the hypotonic buffer, and this volume was pooled with the lysate. Following a brief but vigorous vortex the samples were centrifuged at 14,000 rpm for 10 min at room temperature. The 200 μl supernatants containing soluble or unpolymerized (cytosolic) tubulin were carefully separated from pellets and transferred to separate tubes. The pellets were resuspended in 200 μl of hypotonic buffer containing 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40 and the protease inhibitors described above. Each tube containing either the soluble or the polymerized fraction was mixed with 70 μl of 4× SDS-PAGE sample buffer (0.3 M Tris-HCl, pH 6.8, 45% glycerol, 20% β-mercaptoethanol, 9.2% SDS and 0.04 g/100 ml bromophenol blue) and heated at 95°C for 5-10 min; 20 μl aliquots of each sample then were analyzed by SDS-PAGE on a 12% resolving gel and 3% stacking gel. Following immunoblotting using a primary anti-β-tubulin MAb, the signal was quantitated by densitometry.

Cell Migration Assay

Confluent cultures of HMVEC-L cells were prepared in 24-well plates. A scrape wound of uniform width (2 mm) was produced in the monolayers prior to treatment with CA4DP or drug vehicle. CA4DP exposure was for a period of 2hr at concentrations of 0.1, 1, and 10 μM. The drug was removed and 24 and 48 hr later each well was stained with 300 μg/ml neutral red solution for 30 min to help visualize and localize the cells. The number of cells entering the denuded area was counted using a phase microscope (Braunhut *et al.*, 1996).

Apoptosis

HMVEC-L cells grown in 2-well chamber slides were treated with CA4DP at 1-50 μM for 2 hr. After a specified time, the treated cells were fixed in 4% formaldehyde solution for TUNEL assay. Basically, the cells were permeabilized in 0.2% Triton X-100 solution for 5 min. DNA strand breaks were then labeled with fluorescein-12-dUTP in TdT incubation buffer at 37°C for 1 hr. The samples were then counterstained with 1 μg/ml DAPI, which binds to the A-T-rich regions of DNA. Localized green fluorescence of apoptotic cells (fluorescein-12-dUTP) in a blue background (DAPI) was detected by fluorescence microscopy. The percentage of apoptotic cell was obtained by dividing the number of cells with green fluorescence by the total number of cells with blue fluorescence. A minimum of 300 cells were counted for each condition.

Cell Detachment

HMVEC-l cells were plated in 60 mm petri dishes, and exposed to 0.1-50 μM CA4DP for 2 hr while in logarithmic growth or plateau phase. Cell detachment rates were determined 22 hr after drug treatment by counting the detached cells in the media electronically using a Coulter counter.

Results

Initial studies examined the cellular morphology and tubulin organization of CA4DP-treated cells by immunofluorescence (Figure 3-1). Untreated HMVEC-L cells demonstrated a well-organized tubulin network with individual microtubule fibers clearly visible (Figure 3-1a). Treatment with 10 μM CA4DP for a 4 hr period led to a disrupted network of microtubules, which appears as a diffuse staining pattern in the treated cells (Figure 3-1b). When HMVEC-L cells were treated with 10 μM CA4DP for 24 hr, the tubulin network disruption was even more evident (Figure 3-1c). In some treated cells, tubulin components distributed only in regions around the nucleus, which may account for why cells rounded up and detached from the monolayer (below and Figure 3-6).

In order to quantitate the effects of CA4DP on tubulin polymerization, changes in both soluble and polymerized tubulin levels in HMVEC-L cells treated with CA4DP were examined. Microtubules are an integral part of the cytoskeleton of eukaryotic cells and are composed of two major soluble proteins, α- and β-tubulin. Tubulin exists in cells in two forms, soluble (unpolymerized) tubulin and polymerized (cytoskeletal) tubulin. There exists a dynamic equilibrium between

tubulin polymerization and depolymerization to maintain the normal function of cells. CA4DP possesses a high affinity for the colchicine binding site on tubulin and results from SDS-PAGE analysis showed a time-dependent depolymerization of tubulin when HMVEC-L cells were treated with 5 μM CA4DP (Figure 3-2). Polymerized tubulin comprised ~50% of the total tubulin in HMVEC-L cells before CA4DP exposure. It decreased to 1.3% 24 hr after CA4DP treatment. It should be noted that the effect of CA4DP on tubulin polymerization occurred very rapidly with decreased polymerized tubulin levels being detected within 2 hr after drug exposure.

As non-toxic doses of CA4DP were found to inhibit cell cycle progression (Chapter 2), the effect of CA4DP on endothelial cell migration also was investigated. Endothelial cell migration is an essential endothelial cell function in both angiogenesis and the wound healing process. In angiogenesis, endothelial cells are required to proliferate and migrate in response to angiogenic stimuli (Folkman, 1986). Upon wounding, the cytoskeletal network in a quiescent cell undergoes dramatic redistribution and reorganization to facilitate the directional movement of the cell into the injured area (Braunhut *et al.*, 1996). To mimic this process, a scrape of uniform width (2 mm) was produced in HMVEC-L cell cultures grown to confluency. Cells migrating into the denuded area were counted 24 and 48 hr later. Results revealed that CA4DP-treated HMVEC-L cells exhibited a reduced capacity to migrate as seen by the 10-40% reduction in the number of cells detected in the denuded area (Figure 3-3). In addition, observations made by phase microscopy also showed that, compared to CA4DP-

treated cells, untreated cells could penetrate a greater distance into the denuded area over the same time period.

Although studies using the clonogenic cell survival assay (Chapter 2) revealed a concentration-dependent activity of CA4DP against proliferating HMVEC-L cells (Figure 2-3), this cell survival assay can not distinguish between whether the effects of CA4DP result from cell death by necrosis or apoptosis. To shed light on this issue, HMVEC-L cells were treated with 5 and 50 μM doses of CA4DP for 2 hr and the induction of apoptosis was assessed at various times after treatment by TUNEL assay. Under these conditions, HMVEC-L cells showed clear evidence of nuclear condensation and fragmentation, which are characteristic of apoptosis. When quantified, a time-dependent increase of apoptotic cells in the 50 μM-CA4DP-treated cell population from ~7% immediately after treatment to ~20% 22 hr later was noted (Figure 3-4a). At the lower dose (5 μM), increased apoptosis in HMVEC-L cells could be detected 22 hr after drug treatment. Figure 3-4b also showed that the CA4DP-induction of endothelial cell apoptosis was clearly dose dependent. Compared to only 2% apoptotic cells in the untreated cell population, HMVEC-L cells treated with 50 μM CA4DP for 2 hr exhibited apoptosis levels ~10 fold higher 22 hr after treatment. The result was consistent with previous studies in which DAPI staining was used to visualize the apoptotic cells with nuclear condensation and fragmentation (Table 3-1).

As cells undergoing apoptosis tend to detach from culture dishes, the number of cells in the supernatant after CA4DP treatment were quantitated using a Coulter counter. The results showed a dose-dependent increase in cell detachment when proliferating, but not quiescent, HMVEC-L cell cultures were treated with CA4DP, again demonstrating the selective activity of CA4DP (Figure 3-5). This dose dependent endothelial cell detachment correlated closely with the number of apoptotic cells found on the monolayer (Figure 3-5 vs Figure 3-4b). Furthermore, when the detached cells were examined by TUNEL assay, more than 90% were found to be apoptotic.

Discussion

CA4DP is a tubulin-binding agent which has been shown to produce extensive hemorrhagic necrosis in both rodent and human tumor models (Dark *et al.*, 1997; Li *et al.*, 1998; Horsman *et al.*, 1998). Indeed animal studies with this agent used alone or in combination with traditional anticancer therapies have been sufficiently promising (Li *et al.*, 1998; Chaplin *et al.*, 1999) to initiate Phase I clinical trials with CA4DP in both the UK and US. The preclinical investigations from our laboratory and other groups suggested that the agent's selective activity against proliferating endothelial cells may be of particular importance (Dark *et al.*, 1997). To explore this further, the present studies were undertaken with HMVEC-L cells *in vitro* with the aim of characterizing the effects of CA4DP and delineating more directly the role of the endothelial cell in its mechanism of action.

Strategies aimed at targeting the tumor vessel network, particularly antiangiogenic therapies, have received considerable attention as alternative cancer therapies (Schweigerer, 1995; Denekamp, 1990; Denekamp, 1993). Damaging the tumor vessels directly and selectively with antivascular agents necessitates the existence of key differences between the vessels comprising tumors and normal tissues. The much higher proliferative index of tumor associated endothelial cells as compared to those found in normal tissues provides such a difference (Denekamp, 1993). The development of drugs which are particularly toxic to dividing endothelial cells aim to exploit this difference and offer the possibility of significant treatment selectivity.

Earlier results (Chapter 2) demonstrated that CA4DP acts selectively against proliferating endothelial cells. For example, data in Figure 2-3d illustrated that CA4DP starts to affect the viability of proliferating HMVEC-L cells at the dose ~1 μM. Increasing the drug dose to ~100-fold higher still showd no effect in quiescent HMVEC-L cells. This observation was consistent with previous findings of CA4DP activity in HUVEC cells (Dark *et al.*, 1997). However, as the clonogenic cell survival assay can not distinguish between necrotic and apoptotic cell death, based on the studies from Chapter 2 (Figure 2-4), CA4DP-induced endothelial cell apoptosis was measured in detail. Using the TUNEL assay a dose-dependent induction of apoptosis in HMVEC-L cells after CA4DP treatment could be demonstrated (Figure 3-4). This effect was dramatic at the 50 μM dose, which induced apoptosis in ~20% of the treated endothelial cells that were still attached. Considering the ~10% detached cells that were almost all apoptotic after

50 μM CA4DP treatment, the overall apoptotic cell level was as high as 30%, which was pretty significant. Considering the possibility that only 30-40% of the HMVEC-L cells are actively dividing cells (Chapter 2), we believe that CA4DP induces the death of proliferating HMVEC-L cells predominantly by apoptotic processes. Prior studies using DAPI staining as well as flow cytometric evaluations of HMVEC-L cells stained with annexin-V and propidium iodide, also showed 5-10-fold increases in apoptosis in treated compared to untreated cells (data not shown). These observations are consistent with those of Iyer *et al.* who used increased caspase-3 activity to show that CA4DP induced apoptosis in HUVEC cells (Iyer *et al.*, 1998). It is still possible that the apoptotic cell death may not show the whole picture of the cytotoxicity of CA4DP. Some endothelial cells may die because of losing their clonogenicity after CA4DP treatment. However, since the antivascular effect of CA4DP occurs very rapidly, we believe that for the *in vivo* situation, the induction of apoptosis by CA4DP is the major contributor of cell death compared to clonogenic cell death.

The cytoskeleton of endothelial cells participates in a number of cellular processes, including not only spindle formation and chromosome segregation, but also intracellular transport of molecules, cell motility, and angiogenesis (Giannakakou *et al.*, 1998). The microtubules which form an integral part of the cytoskeleton therefore provide an attractive molecular target for anti-vascular drugs such as CA4DP. The present studies showed that CA4DP caused a time-dependent tubulin depolymerization in HMVEC-L cells. Within 24 hr, most microtubules in the cells depolymerized into free tubulin subunits (Figure 3-2).

Immunofluorescence studies showed complementary results; a disorganized pattern of microtubules was evident in CA4DP-treated HMVEC-L cells rather than a normal cytoskeletal architecture (Figure 3-1). These findings indicate that CA4DP binds to tubulin and shifts the dynamic equilibrium that normally exists in cells between polymerized and soluble tubulin.

Disrupted microtubule structure also directly affects the ability of cells to migrate. Figure 3-3 shows that CA4DP doses of 0.1 ~ 10 μM inhibited, by 10 ~ 40%, the ability of HMVEC-L cells to migrate into denuded areas in culture plates. These findings suggest that, although CA4DP may be predominantly acting as an antivascular agent, it also possesses at least some of the features typically associated with antiangiogenic agents, namely effects on migration and proliferation. This conclusion is consistent with the results from Chapter 2 which showed that CA4DP at doses less than those affecting cell survival in the clonogenic cell survival assay, blocked HMVEC-L cells at the G_2/M phase of the cell cycle (Figure 2-2).

Another outcome of microtubule structure disruption is endothelial cell detachment. Results from Figure 3-5 showed that CA4DP caused a dose-dependent cell detachment in proliferating HMVEC-l cells. Although not shown, at high doses, cells began to round up and detach immediately after a 2-hr CA4DP treatment. If such an effect occurs *in vivo*, it could explain the vascular shutdown seen after CA4DP exposure. Considering the irregular, capillary-like vessels in tumors, even if only a few endothelial cells round up and detach from the

monolayer vessel bed, this might be sufficient to occlude the blood flow and ultimately shut down the whole vascular supply in the tumor.

In summary, the present results show that CA4DP is specifically toxic to proliferating endothelial cells predominantly by apoptotic pathways. CA4DP also inhibits tubulin polymerization, endothelial cell migration and attachment. While the *in vitro* results indicate that CA4DP has potent effects on endothelial cells, it should be recognized that vascular shutdown in CA4DP-treated tumors can occur within 20 min after treatment (Sackett, 1993; Li *et al.*, 1998). The rapidity of vascular shutdown observed suggests that more immediate changes are responsible for the drug effects seen. One possibility is that CA4DP can have dramatic effects on the three-dimensional shape of newly formed endothelial cells. It is possible that the early manifestations of cell shape changes brought on by CA4DP effects on tubulin binding which lead to cell detachment and apoptosis *in vitro*, result in similar physical effects *in vivo* which dramatically alter capillary blood flow, expose basement membrane and, as a result, induce haemorrhage and coagulation. Recent studies showed that CA4DP induces endothelial shape changes with a consequent increase in permeability of an endothelial cell monolayer to macromolecules (Twardowski and Gradishar, 1997). The increase in vascular permeability to macromolecules may result in an increase in interstitial fluid pressure, an increase in blood viscosity, procoagulative effects, vascular collapse, and the induction of cytokines.

The present findings provide a basis for the selective action of CA4DP against the proliferating endothelial cell population found in tumors. The reason

for the tumor selectivity of CA4DP may relate to differences in proliferation rate of endothelial cells in tumors and normal tissues. Further investigations of morphological changes of endothelial cells *in vivo* need to be pursued.

a.

b.

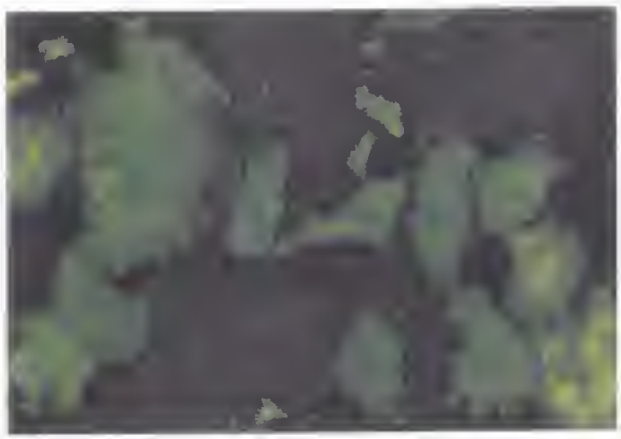


Figure 3-1. Indirect immunofluorescence of microtubules in HMVEC-L cells. a) No drug treatment; b) 4 hr treatment with 10 μM CA4DP.

c.

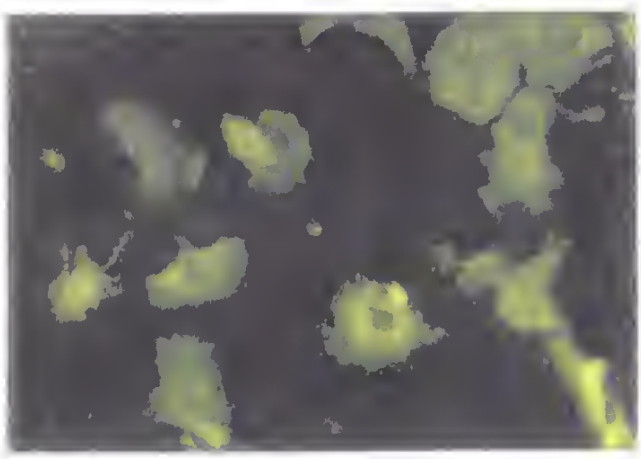


Figure 3-1-continued. c) 24 hr treatment with 10 µM CA4DP.

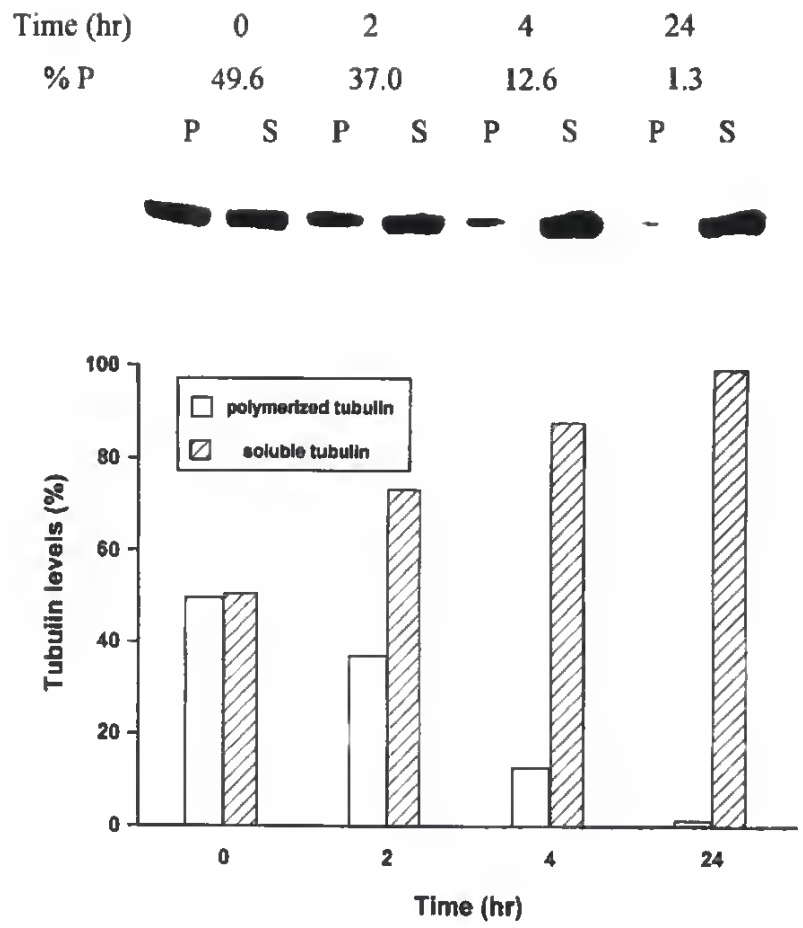


Figure 3-2. Effect of CA4DP on tubulin polymerization in HMVEC-L cells. The cells were treated with 5 μM CA4DP over a 24-hr period. Cells were harvested at different time points, and tubulin polymerization was assessed. The percent of polymerized tubulin (%P) was determined by dividing the value of polymerized tubulin by the total tubulin content (the sum of P and S).

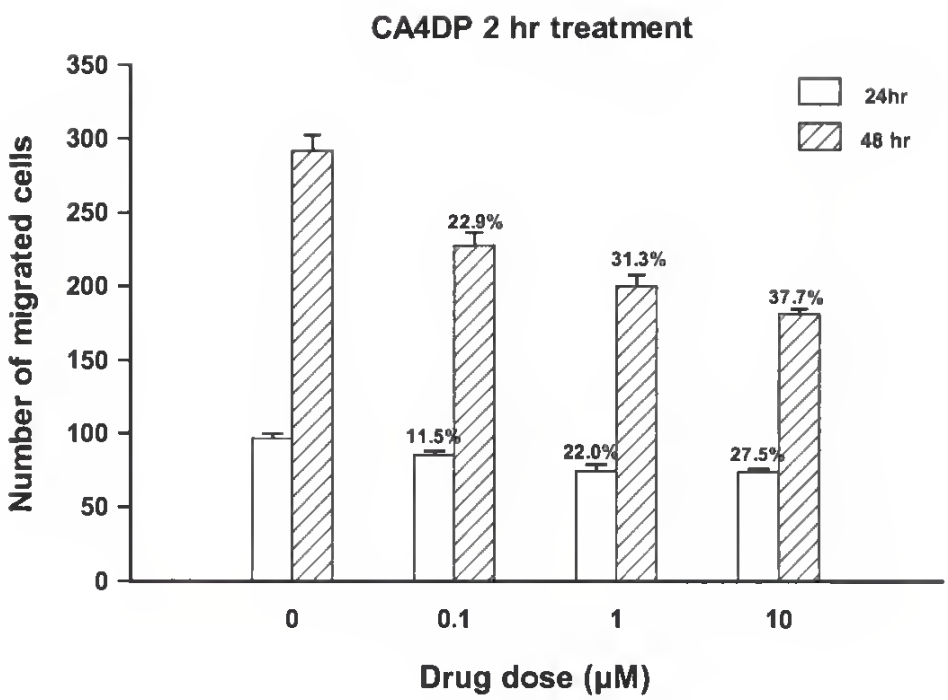
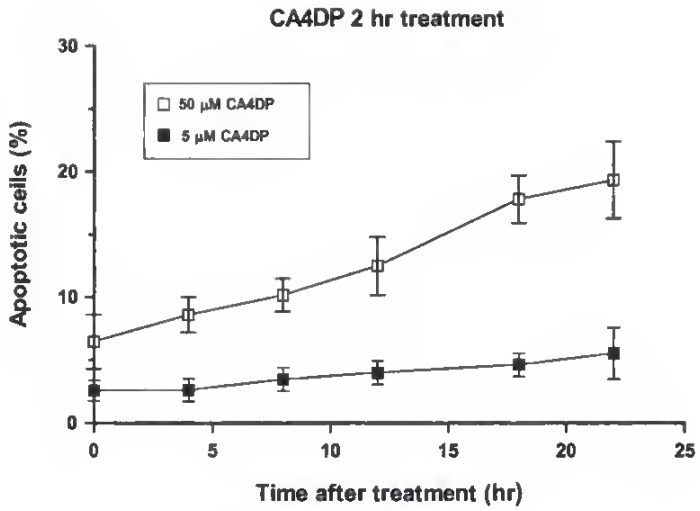


Figure 3-3. HMVEC-L cell migration into a 2 mm denuded area in culture plates. Cells were either untreated or exposed to CA4DP for 2 hr. Migrating cell numbers were counted 24 and 48 hr later. Data are the mean ± SE of 6 replicates.

a.



b.

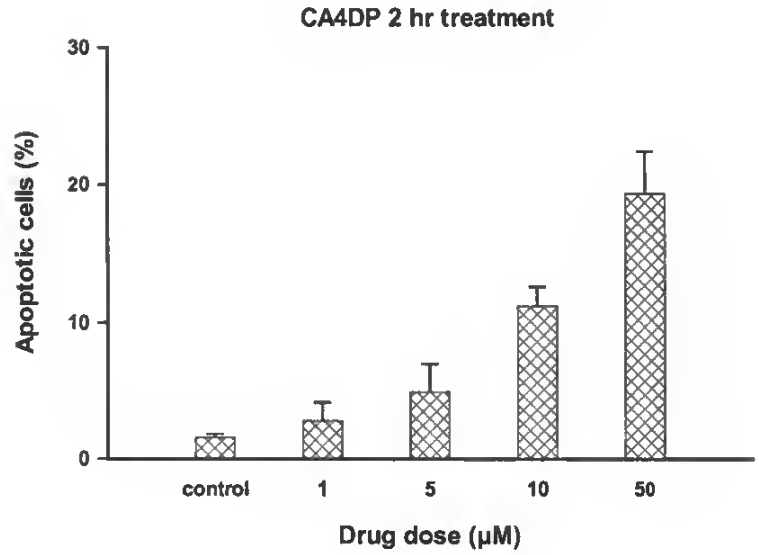


Figure 3-4. Effect of CA4DP on HMVEC-L apoptosis. (a) HMVEC-L cells were treated with 5 and 50 μM CA4DP for 2 hr and stained for TUNEL assay after a specified time. (b) HMVEC-L cells were treated with various doses of CA4DP for 2 hr, and the apoptotic nuclei in the monolayer were counted 22 hr later. Data are the mean ± SE of three independent experiments.

Table 3-1. COMPARIOSN OF APOPTOSIS DATA FROM TUNEL ASSAY AND DAPI STAINING (CA4DP 2 HR TREATMENT)

	APOPTOTIC CELLS (%)	
TREATMENT	TUNEL	DAPI
control	1.8 (± 0.2)	1.8 (± 0.2)
1 μM	3.4 (± 0.7)	4.9 (± 1.4)
5 μM	5.6 (± 1.9)	7.2 (± 2.1)
10 μM	11.8 (± 1.4)	13.3 (± 1.6)
50 μM	19.6 (± 3.0)	19.4 (± 2.7)

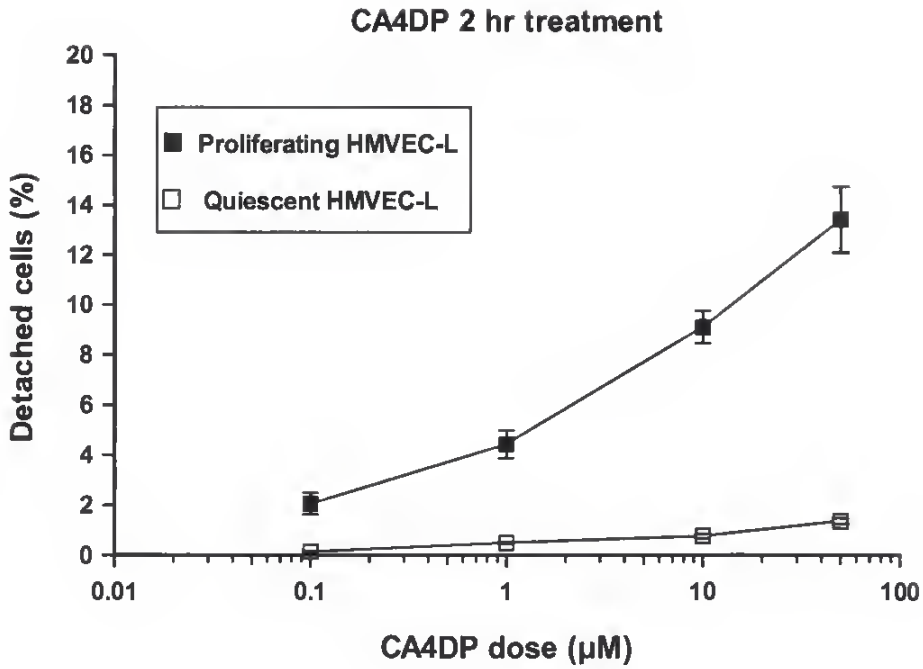


Figure 3-5. Quantification of cell detachment in CA4DP treated HMVEC-L cells. Cells were treated with various doses of CA4DP for 2hr and the numbers of detached cells were counted electronically 22 hr later. Data are the mean ± SE of 6 replicates.

CHAPTER 4
STUDY OF THE EFFECTS OF CA4DP IN THE MODEL OF KAPOSI'S SARCOMA

Introduction

Kaposi's Sarcoma (KS) is a highly vascularized neoplasm that primarily results in raised, highly vascularized lesions (Groopman, 1987; Tappero *et al.*, 1993). Before the 1980s, KS was a rare disorder that occurred predominantly in elderly men of Mediterranean or Eastern European Jewish descent. With the advent of the acquired immunodeficiency syndrome (AIDS) epidemic, its occurrence has increased dramatically. KS is classified into four different types: classic, African endemic, iatrogenic or drug-associated, and AIDS-related (Sung *et al.*, 1997). Classic KS usually follows an indolent and benign clinical course that rarely requires treatment. In contrast, AIDS-KS is a fulminant disease that requires aggressive pharmacotherapy, especially when it involves visceral organs.

AIDS-KS is the most common neoplastic disease in patients with AIDS. Presently, it is the fourth leading clinical manifestation of AIDS (Pluda *et al.*, 1993). Cutaneous or mucocutaneous lesions may occur. Lesions occurring in the viscera primarily affect the gastrointestinal tract, lymph nodes, and pulmonary system. Shortness of breath, dyspnea on exertion, increased respiratory rate, and decreased oxygen saturation are common in patients with pulmonary involvement. Involvement of the pulmonary tract occurs in 15-50% of patients

and is estimated to contribute to 25% of deaths in patients with AIDS-KS (Tappero *et al.*, 1993).

Histopathology of KS reveal highly vascularized lesions with abundant angiogenesis accompanied by abnormal blood vessel development and leakage of blood (Gallo, 1998). The predominant cells in the tumor are the spindle-shaped cells believed to be the tumor cells of KS. These cells express some of the surface markers of activated endothelium but also contain smooth-muscle actin, suggesting that the cell of origin may be a primitive vascular cell (Kroll and Shandera, 1998). Growth factors that support spindle cell proliferation include interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) (Sung *et al.*, 1997). Proteins that regulate neovascularization or angiogenesis such as basic fibroblast growth factor (bFGF), platelet-derived growth factors, and vascular endothelial growth factor (VEGF) also promote growth of spindle cells (Nakamura *et al.*, 1992).

Standard treatments for KS include intralesional injection of vinblastine or α_2-interferon, local radiotherapy and systemic chemotherapy. These treatments are administered primarily for symptomatic relief and to prevent disease progression. Cytotoxic chemotherapy is the standard therapy for patients with extensive lesions and disease involving visceral organs and lymph nodes. A single agent such as vincristine or α_2-interferon is used for mild cases. More advanced cutaneous or visceral KS is usually treated with combination chemotherapy including agents such as vincristine, bleomycin, and doxorubicin. However, these treatments do not significantly prolong survival, and the clinical effectiveness is

not satisfactory (Lilenbaum and Ratner, 1994). Vincristine or α_2-interferon monotherapy give a clinical response in only about 25% of patients, while the combination of vincristine, bleomycin, and doxorubicin causes regression in about 40% (Kroll and Shandera, 1998). Continued pursuit of more effective and less toxic agents is clearly needed.

Given the tumor's histopathology, a variety of new treatment approaches, particularly those focused on inhibiting tumor angiogenesis are being investigated. Angiogenesis plays a crucial role in the pathogenesis and progression of KS (Cornali *et al.*, 1996) and antiangiogenesis approaches may provide a means of arresting the progression of KS. The drug initially tested as an angiogenesis inhibitor in patients was TNP-470. When administered once weekly by intravenous infusion, this agent gave partial responses in KS patients (Dezube *et al.*, 1998). More recently, there has been an interest in exploring the clinical utility of thalidomide as an anti-KS agent. This was based on evidence that thalidomide could inhibit angiogenesis, block tumor necrosis factor alpha (TNF-α), and inhibit intercellular adhesion molecules and basement membrane formation (Gascón and Schwarts, 2000). Preliminary results from two Phase II clinical trials showed that thalidomide had activity in a subset of patients with KS (Welles *et al.*, 1997; Bower *et al.*, 1997). Based on the encouraging results observed with thalidomide and TNP-470, clinical research into the antiangiogenic activities of these and other agents, including interleukin-12 and angiostatin, continues (Kroll and Shandera, 1998; Gascón and Schwarts, 2000).

Because KS is a highly vasoactive neoplasm, directly targeting the actively growing vessels of the tumor may be another approach suitable for KS treatment. Since large numbers of neoplastic cells are directly supported by small numbers of endothelial cells, damaging the tumor endothelium could have marked impact on tumor cell survival and growth (Bicknell and Harris, 1992). In the present investigation we examined the efficacy of CA4DP in KS xenografts by assessing the extent of both vascular damage and cytotoxic action in these tumors.

Methods and Materials

KS Xenografts and Treatments

KS xenografts were initiated by injecting the flanks of 6-8-week-old athymic NCR nu/nu mice (Frederick Laboratories, Frederick, MD) with 1×10^6 KSY-1 cells (ATCC, Rockville, MD) (Lunardi-Iskandar *et al.*, 1995) and were serially passed by subcutaneous transplantation of tumor pieces in the flanks. Macroscopic tumors were available for experiments 3-4 weeks later. Tumor-bearing mice were allocated to groups and received either no treatment or different doses of CA4DP (OXiGENE Inc., Lund, Sweden). CA4DP was dissolved in 0.9% sterile saline and injected intraperitoneally in a volume of 0.01 ml/g animal body weight.

Hoechst-33342 Studies

Hoechst-33342 (bisBenzimide, Sigma) solution was made up in 0.9% sterile saline immediately before use. KS-bearing mice were either untreated or treated with 100 mg/kg CA4DP. Hoechst-33342 then was administered at 40 mg/kg

intravenously (volume 5 ml/kg) at various times after CA4DP injection (Smith *et al*, 1988). One minute after Hoechst-33342 injection the mice were killed, the tumors and the normal tissues (lung, liver, and muscle) of the mice were resected and immediately immersed in liquid nitrogen for subsequent frozen sectioning. For each tumor sample, 10 μm cryostat sections were cut at three different levels between one pole and the equatorial plane. The sections were air dried and then studied under UV illumination using a fluorescent microscope. Blood vessel outlines were identified by the surrounding halo of fluorescent H33342-labelled cells. Vessel counts were performed using a Chalkley point array for random sample analysis (Curtis, 1960). Briefly, each section was viewed at ×10 objective magnification. A 25-point Chalkley grid was positioned randomly over field of view. Any points falling within haloes of fluorescent cells were scored positive. Twenty random fields were counted per section and a minimum of six sections per tumor was examined.

Histological Staining

Histological sections were prepared from KS xenografts 4 and 24 hr after CA4DP was given. All specimens were fixed in 10% neutral buffered formalin, routinely processed and embedded in parafin. Sections (4 μ) applied to slides were deparaffinized in xylene and hydrated through graded alcohols. Standard hematoxylin and eosin (H&E) staining was used for each slide. Necrotic fractions in KS tumors were quantified by image analysis. Briefly, stained sections were divided into 4-8 grids and areas of necrosis within each grid were traced on an

Image Pro Plus system. All grid measurements were combined and the percentage of necrosis relative to the total area of the tumor was calculated.

Clonogenic Cell Survival Assay

Clonogenic cell survival in treated or untreated tumors was assessed using an *in vivo* to *in vitro* clonogenic cell survival assay as previously described (Allalunis-Turner and Siemann, 1986; Siemann, 1995). Briefly, 24 hr after treatment, tumor-bearing mice were killed and their tumors excised and then dissociated to a single cell suspension using an enzyme cocktail (0.025% collagenase, 0.05% pronase, and 0.04% DNase). The cells then were mixed with 10^4 lethally irradiated cells in α-MEM medium containing 10% fetal calf serum and plated into 60 mm petri dishes. After 2 weeks of incubation at 37°C, colonies of 50 or more cells were counted with the aid of a dissecting microscope. Tumor surviving fractions were determined by multiplying the calculated fraction of surviving cells by the ratio of cells recovered in treated versus untreated tumors.

Tumor Growth Delay Assay

Once KS xenografts had reached a minimum size of 200 mm^3, the mice were allocated to different groups for CA4DP treatment. A 100 or a 300 mg/kg dose of CA4DP was used in the single-dose treatment studies. For the multiple-dose groups, 100 mg/kg CA4DP was administered either on days 1, 3, and 5 or on days 1, 5, and 9. After treatment, tumors were measured daily using calipers and the perpendicular diameters were determined. Tumor volumes were estimated using the equation: Volume = $4\pi r^3/3$, $r = (a+b)/4$, where a and b are the perpendicular diameters. The time for each tumor to reach a size of 900 mm^3 was recorded.

Hypoxia Stress and CA4DP Treatment

KSY-1 cells were plated into 60 mm petri dishes at 2×10^5 cells/dish. Once KSY-1 cells were attached to the dishes, the cells were treated with a range of doses of CA4DP. Treated KSY-1 cells used for hypoxic conditions were immediately placed in an airtight chamber and subjected to repeated rounds of evacuation and replacement with nitrogen gas. The sealed chambers were then incubated for 24 hr at 37°C. KSY-1 cells under aerobic conditions were also treated with a range of doses of CA4DP at 37°C for 24 hr. After CA4DP treatment, these treated cells were then trypsinized, counted, and plated into 60 mm $Cell^+$ petri dishes for clonogenic cell survival assay. After 2 weeks of incubation at 37°C, colonies of 50 or more cells were counted with the aid of a dissecting microscope. Cell surviving fractions were calculated as the ratio of colonies counted in treated versus untreated group.

Results

Initial studies focused on the early effects of CA4DP treatment on tumor vasculature. Results obtained with the Hoechst-33342 fluorescent dye showed that a single dose of 100 mg/kg CA4DP caused an almost complete vascular shutdown in KS xenografts within 4 hr after treatment (Figure 4-1). Compared to the abundant vasculature in the untreated tumors, KS xenografts in mice treated with CA4DP showed vessels essentially only near the periphery of the tumors. This vascular damaging effect of CA4DP occurred rapidly and was detected in KS xenografts 30 min after drug treatment. Indeed, when functional vessels were counted, most were found to be shutdown by CA4DP within 0.5 to 2 hr after

treatment (Figure 4-2). The vasculature in normal tissues from untreated and CA4DP-treated mice also were assessed. It is clearly seen from Figure 4-3 that none of the vascular networks in the lung, the liver, and the muscle was affected at 4 hr after a 100 mg/kg dose of CA4DP treatment.

Histological evaluations of KS xenografts showed morphological evidence of damage in tumor cells within a few hours after CA4DP treatment. By 24 hr after CA4DP treatment (100 mg/kg), extensive haemorrhagic necrosis could be seen with viable tumor cells detectable only at the periphery of the tumor adjacent to the surrounding normal tissues (Figure 4-4).

To quantify the extent of necrosis produced by CA4DP treatment, sections from KS xenografts removed 24 hr after treatment were assessed using an image analysis system. The results (Figure 4-5) showed that compared to the ~10% necrosis seen in untreated tumors, treatment with 50 mg/kg CA4DP increased the extent of necrosis to ~60%. In xenografts treated with 100 mg/kg CA4DP, the necrotic fraction increased to ~90% 24 hr after drug treatment.

Anti-tumor effectiveness of CA4DP was determined by measuring clonogenic cell survival in KS xenografts treated with various doses of this agent. The data demonstrated that administering increasing doses of CA4DP to tumor-bearing mice resulted in a dose-dependent increase in tumor cell kill (Figure 4-6). A comparison of results in Figures 4-5 and 4-6 further shows a consistency between the clonogenic cell survival data and the results of the histological evaluations, i.e. a 100 mg/kg dose of CA4DP caused ~90% tumor cell death and necrosis 24 hr after treatment. The tumor cell killing effect with a single 100

mg/kg dose of CA4DP treatment also was assessed at various times after treatment. The results showed that the maximum tumor cell kill (>90%) was achieved between 1-3 days after CA4DP treatment (Figure 4-7). At later times cell survival recovered.

Hypoxic tumor cells normally exist in central areas of tumors, far away from sources of efficient oxygen supply. Since CA4DP causes extensive central necrosis in KS xenografts, it raised the question of whether part of the effect of this agent was the consequence of CA4DP killing hypoxic cells more efficiently than well-oxygenated tumor cells. To answer this question, we examined the toxicity of CA4DP in both aerobic and hypoxic KSY-1 cells *in vitro* (Figure 4-8). The results showed that hypoxic KSY-1 cells were not more susceptible to CA4DP treatment than aerobic KSY-1 cells. Indeed clonogenic cell survival determinations showed the surviving fraction of hypoxic cells after CA4DP treatment to be slightly higher than that for aerobic cells. This result further supports the notion that it is the antivascular effects of CA4DP, not a higher sensitivity of hypoxic tumor cells to CA4DP, that are responsible for the induced extensive central necrosis in KS tumors after CA4DP treatment.

In conjunction with the cell survival investigations, studies evaluating the effects of CA4DP on KS growth also were performed. Tumor-bearing mice were treated at a size of 200 mm^3 with a single dose of either 100 or 300 mg/kg dose of CA4DP. Such treatments resulted in a slight, but not significant, tumor growth delay (Figure 4-9, Table 4-1). Indeed the higher dose of CA4DP (300 mg/kg) did not increase the tumor growth delay compared to that achieved with 100 mg/kg.

In an attempt to apply this agent more efficiently and in particular to impair the regrowth of vasculature from the surviving rim of tumor cells (Figures 4-1 and 4-4), studies utilizing repeated injections of CA4DP (100 mg/kg) also were performed. Treatment commenced when the tumors reached 200 mm^3. Two different treatment schedules were used: CA4DP was administered on days 1, 3 and 5 or on days 1, 5, and 9. The results showed a far superior response of the KS xenografts to the multiple CA4DP treatment schedules (Figure 4-9). Both treatments induced significant growth delay compared to untreated tumors and tumors treated with either single dose of CA4DP (Table 4-1). Administering CA4DP using the multiple dose schedule resulted in a growth delay of ~20 days (Figure 4-9, Table 4-1).

Discussion

Kaposi's sarcoma is the most common tumor seen in HIV-infected patients. Several chemotherapeutic agents including vinca alkaloids, etoposide, bleomycin, and doxorubicin are commonly used to treat AID-KS patients (Sung *et al.*, 1997). Vinca alkaloids (either vincrinstine, vinblastine, or an alternating regimen of the two) exert their anticancer effect by binding to tubulin and preventing its polymerization to form microtubules (Chabner and Collins, 1990; Yarchoan, 1999), thus inhibiting a number of cellular processes, including mitosis. Over the years there have been several advances in the therapy of this disease, including the use of liposomal anthracyclines, paclitaxel, and antiangiogenesis agents TNP-470 and thalidomide (Yarchoan, 1999; McGarvey *et al.*, 1998). Among these, paclitaxel, which also interferes with microtubule dynamics by promoting the

formation of highly stable microtubules which resist depolymerization, was found to inhibit the growth of KS-derived spindle cells and to be a potent inhibitor of endothelial cell proliferation (Saville *et al.*, 1995).

Like vinca alkaloids, CA4DP also binds to tubulin and inhibits tubulin polymerization. However, unlike the vinca alkaloids, CA4DP has demonstrated antivascular effects at very low doses (Dark *et al.*, 1997; Li *et al.*, 1998; Chaplin *et al.*, 1999). It has been well established that the vascularization of solid tumors is a prerequisite if a clinically relevant size is to be reached (Folkman, 1986). The dependence of the tumor on its induced vessel formation has created a great deal of enthusiasm in specifically targeting the microcirculation in cancer therapy (Denekamp, 1993). Results from our laboratories and those of others have shown not only that CA4DP has specific effects on actively dividing endothelial cells but also that this agent can cause rapid vascular shutdown in a variety of preclinical tumor models (Dark *et al.*, 1997; Li *et al.*, 1998; Horsman *et al.*, 1998). Because KS is a highly vascularized neoplasm with a cellular origin suggested to be endothelial cell derived, and because tubulin-binding agents have previously been found to be active in KS, the present study was undertaken to examine the efficacy of CA4DP in an *in situ* model of this disease.

The KSY-1 cell line originated from cells isolated from the pleural effusion of an AIDS-associated KS patient (Lunardi-Iskandar *et al.*, 1995). KSY-1 cells promote tumorigenesis, angiogenesis, and metastasis in immunodeficient mice. The model's similar biological, morphological and immunophenotype make it a valuable adjunct for studies related to pathogenesis and therapy of AIDS-KS

(Rojiani *et al.*, 2000). In the present study, KSY-1 cells were used to initiate KS xenografts in athymic mice in order to assess their response to the vascular targeting agent CA4DP.

The pathophysiological effects of CA4DP observed in the present study in KS xenografts were similar to those previously reported by our laboratory in the rodent KHT sarcoma model (Li *et al.*, 1998). CA4DP treatment resulted in a rapid induction of vascular damage in tumors such that 4 hr after treatment there existed an almost complete vascular shutdown (Figures 4-1 and 4-2). This was followed by extensive secondary tumor cell death due to ischemia (Figures 4-4 and 4-5). Histological assessments showed extensive haemorrhagic necrosis 24 hr after CA4DP was administered systemically to KS-bearing mice, with only a small rim of viable tumor cells surviving near the periphery of the tumor (Figure 4-4). These tumor cells probably survived because they were close to the surrounding normal tissues where they were supplied with nutrients from the normal tissue vasculature which was not affected by the action of CA4DP. Studies with intravital microscopy have shown that peripheral tumor tissue retains some blood flow after CA4DP treatment but becomes hemorrhagic with dilated blood vessels (Tozer *et al.*, 1999). This suggests that vascular permeability changes may be more profound in the periphery, where most of the extravasation of macromolecules occurs under unperturbed conditions.

Results from the clonogenic cell survival investigations were consistent with the histological observations. For example, a 100 mg/kg dose of CA4DP which induced ~90% necrosis in KS xenografts also reduced the viable tumor cell

population to ~10% of the pretreatment value (Figures 4-5 and 4-6). The cell survival evaluations established that CA4DP treatments led to a concentration-dependent killing of KS tumor cells (Figure 4-6). This killing manifested itself primarily as a rapid loss of viable cells from the cell population within the 24-hr period after CA4DP treatment. Although normal tissue toxicities were not measured in the present studies, the antivascular effects of CA4DP were achieved at doses less than 1/10 of the maximum tolerated dose (MTD) and without detectable morbidity as previously reported (Dark *et al.*, 1997). While some of the other tubulin binding compounds, for example the vinca alkaloids, may also express antivascular action, they exert their effects at doses approaching the MTD and often only in the presence of significant morbidity (Chaplin *et al.*, 1996). Indeed vinca alkaloid therapy in AIDS-KS patients is frequently limited by neutropenia and peripheral neuropathy (Sung *et al.*, 1997). CA4DP's high tubulin binding affinity (Pettit *et al.*, 1989), selective toxicity in proliferating endothelial cells, and effectiveness at low doses (Dark *et al.*, 1997) may prove to be of considerable value in the continuing clinical evaluation of CA4DP as an antivascular agent.

The present studies showed that a single 100 mg/kg dose of CA4DP had little effect on the growth of KS xenografts (Figure 4-6) despite the fact that this dose of CA4DP causes extensive central necrosis in these tumors (Figures 4-4 and 4-5). The most likely explanation is that the remaining viable tumor cells, located at the periphery of the tumor near the normal tissue, survive and continue to proliferate. This conclusion is supported by the study illustrated in Figure 4-7.

The viable tumor cell fraction began to increase 2 days after CA4DP treatment because of the continued proliferation of cells at the rim of the tumors. The lack of a change in tumor growth following the 100 mg/kg CA4DP treatment probably is a consequence of a balance between the growth of new cells from the surviving rim and the removal of necrotic material from the tumor's core. Increasing the single dose to 300 mg/kg did not result in a greater growth delay in KS xenografts (Figure 4-9). However this result is not surprising given the extent of cell death and necrosis caused by a 100 mg/kg CA4DP dose (Figures 4-5 and 4-6). Increasing the dose further would have little additional effect on the tumor cells which survive due to their location near normal blood vessels.

The effect of multiple exposures of CA4DP on KS xenograft growth also was examined. The rationale for these studies was two-fold. First, it was apparent from the single dose studies that maximum anti-tumor efficacy occurred with doses of ~100 mg/kg (Figures 4-5 and 4-6) and that little could be gained by increasing the exposure dose further. Second, and more importantly, we reasoned that administering multiple doses of CA4DP at times when the tumor is regrowing and the tumor vasculature is recovering and/or re-establishing itself might prove to be a much more efficient application of this agent. From Figure 4-7 we know that it was between 2 and 5 days after single dose of CA4DP treatment that the KS tumors showed recovery and regrowth. Therefore, two different treatment schedules, either giving CA4DP on days 1, 3, and 5 or on days 1, 5, and 9, were examined in KS-bearing nude mice. The results showed that unlike the single dose treatments, both multiple dose schedules caused significant growth delay in

the xenografts (Figure 4-9 and Table 4-1). Multiple doses of CA4DP were clearly far more effective at inhibiting KS growth than single dose treatments. For example, administering three 100 mg/kg dose fractions of CA4DP, as opposed to a single 300 mg/kg treatment, increased the growth delay by ~14-17 days (Table 4-1). To date we have not optimized these multiple CA4DP treatment schedules in the KS model. Whether similar gains can be achieved with lower doses/fraction and/or greater numbers of multiple treatments is currently under investigation. Still, the studies described in the present investigations indicate that administering multiple doses of CA4DP is a very effective way of inhibiting KS growth.

In conclusion, CA4DP treatment can cause vascular shutdown, haemorrhagic necrosis, extensive tumor cell killing, and growth delay in KS xenografts. These findings suggest a possible application of the vascular targeting agent CA4DP in the clinical management of KS.

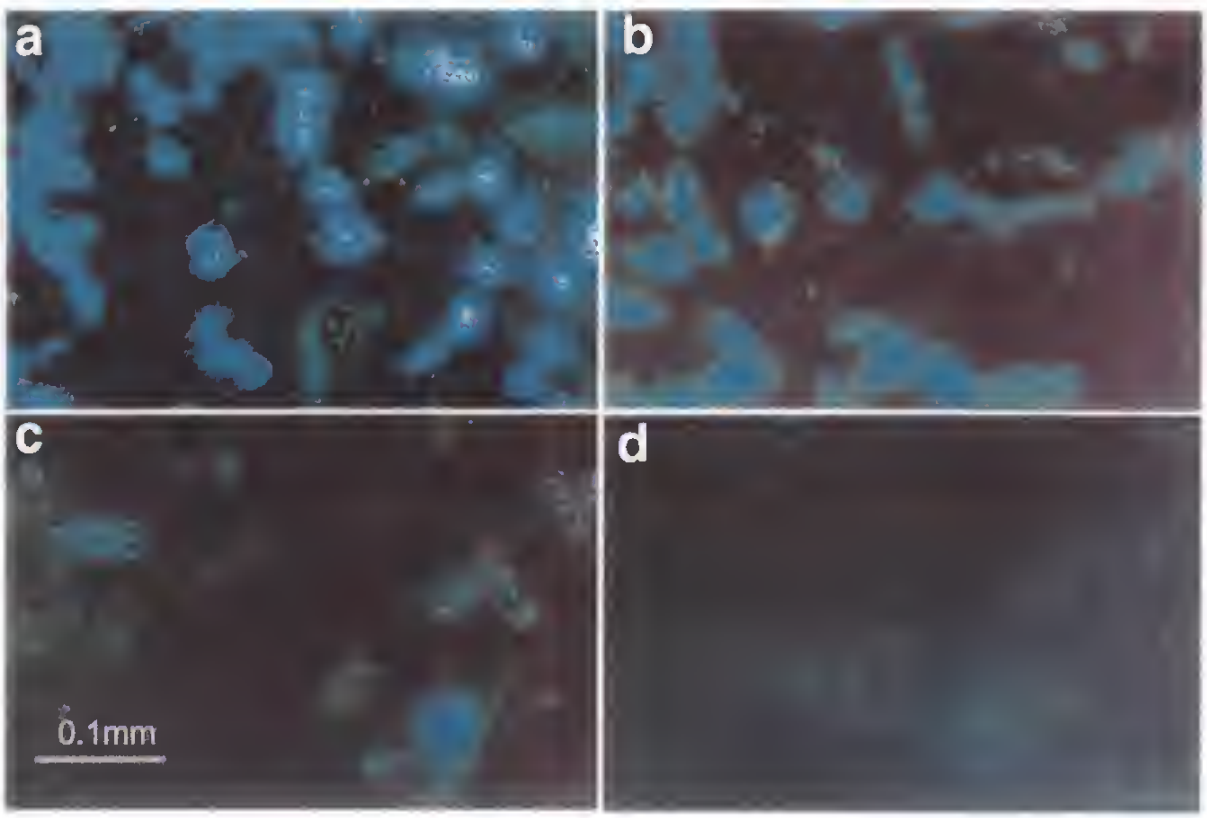


Figure 4-1. KS tumors removed 1 min after i.v. injection of 40 mg/kg H-33342. Vessels were identified by the surrounding fluorescent tumor cells. Tumors were either from untreated mice (a) or from mice 30 min (b), 2 hr (c), and 4 hr (d) after a 100 mg/kg CA4DP treatment. Magnification was ×32 for a-d.

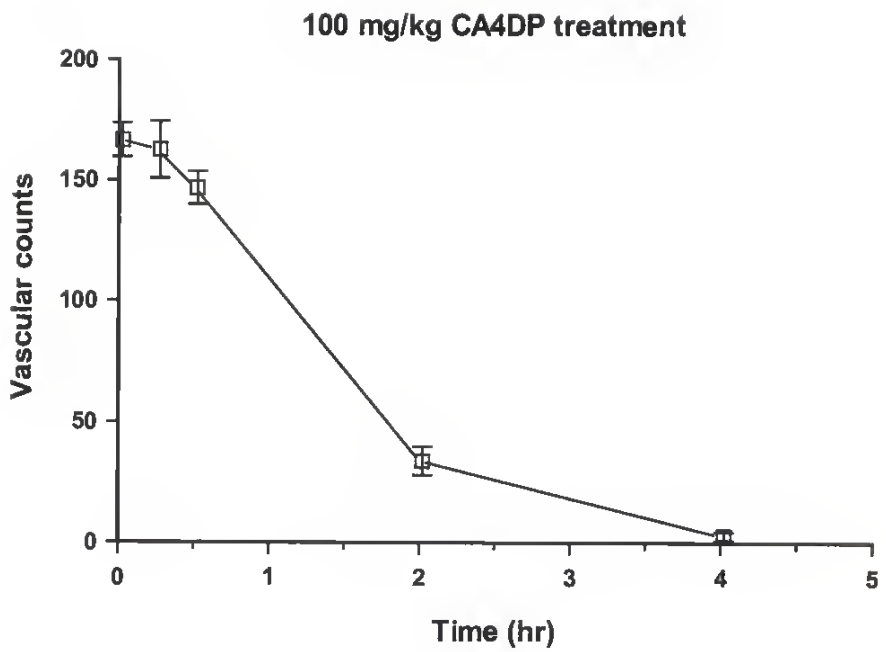


Figure 4-2. Vascular counts for KS tumors with Hoechst-33342 staining. Tumor-bearing mice were treated with 100 mg/kg CA4DP for a specified time before i.v. injection of 40 mg/kg Hoechst-33342. Vessel counts were performed by using a Chalkley point array for random sample analysis. Data are the mean ± SE.

A.

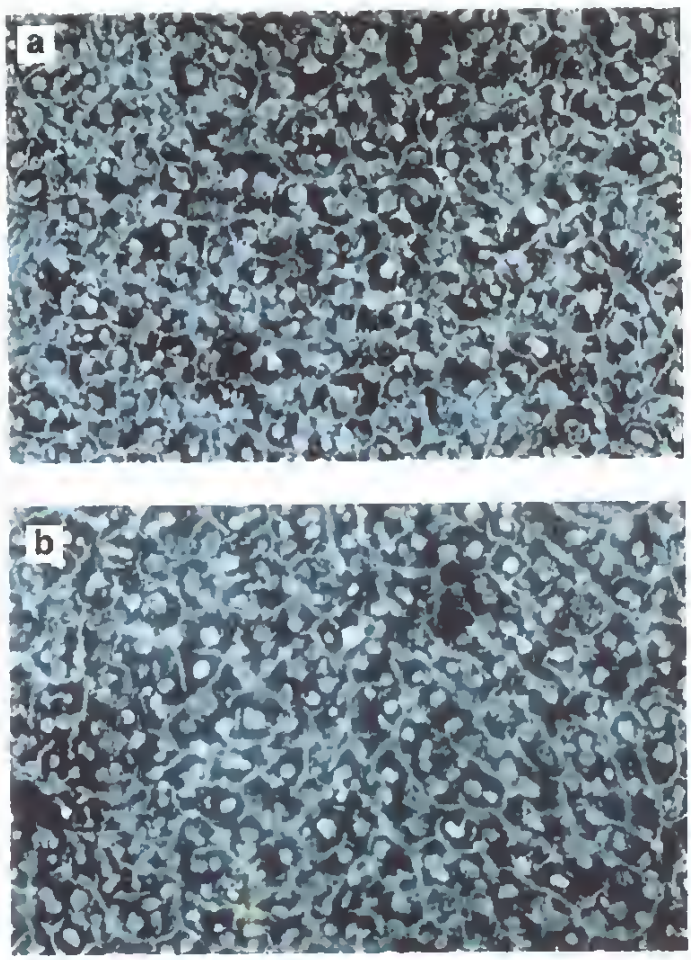


Figure 4-3. Normal tissue sections from KS-bearing mice removed 1 min after i.v. injection of 40 mg/kg H-33342. Vessels were identified by the surrounding fluorescent cells. Samples were either from untreated mice (a) or from mice 4 hr after a 100 mg/kg CA4DP treatment (b). Magnification was ×10. A) Cryostat sections of the liver.

B.

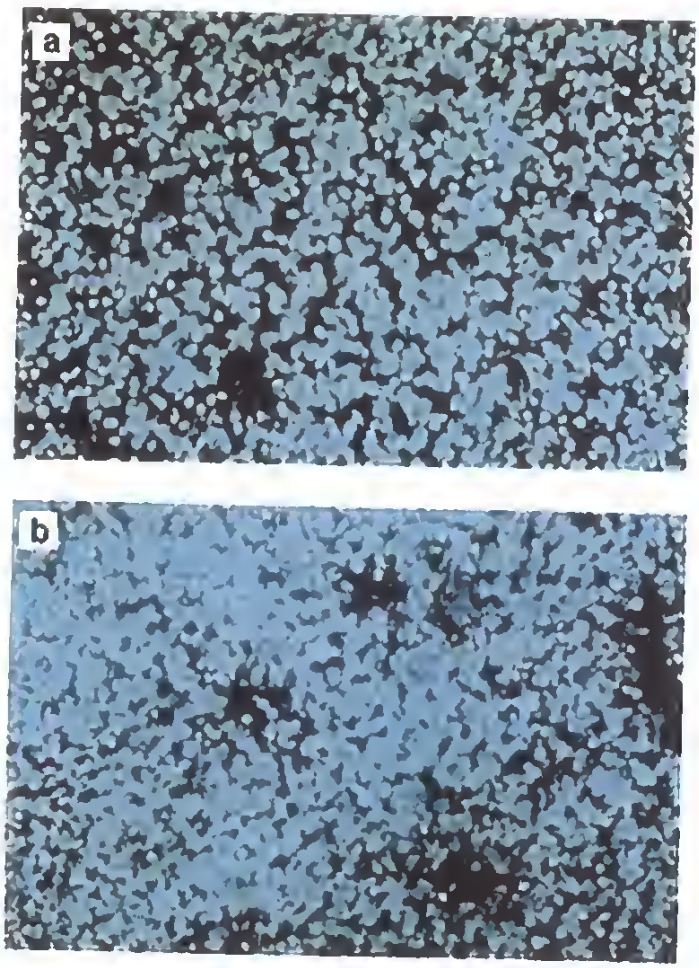


Figure 4-3-continued. B) Cryostat sections of the lung.

C.

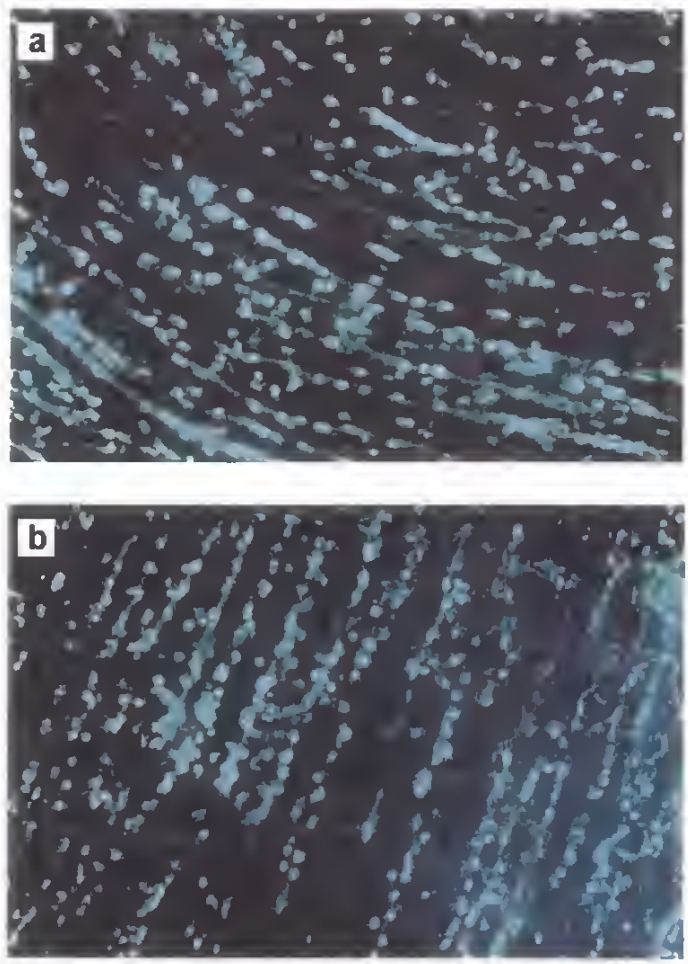


Figure 4-3-continued. C) Cryostat sections of the leg muscle.

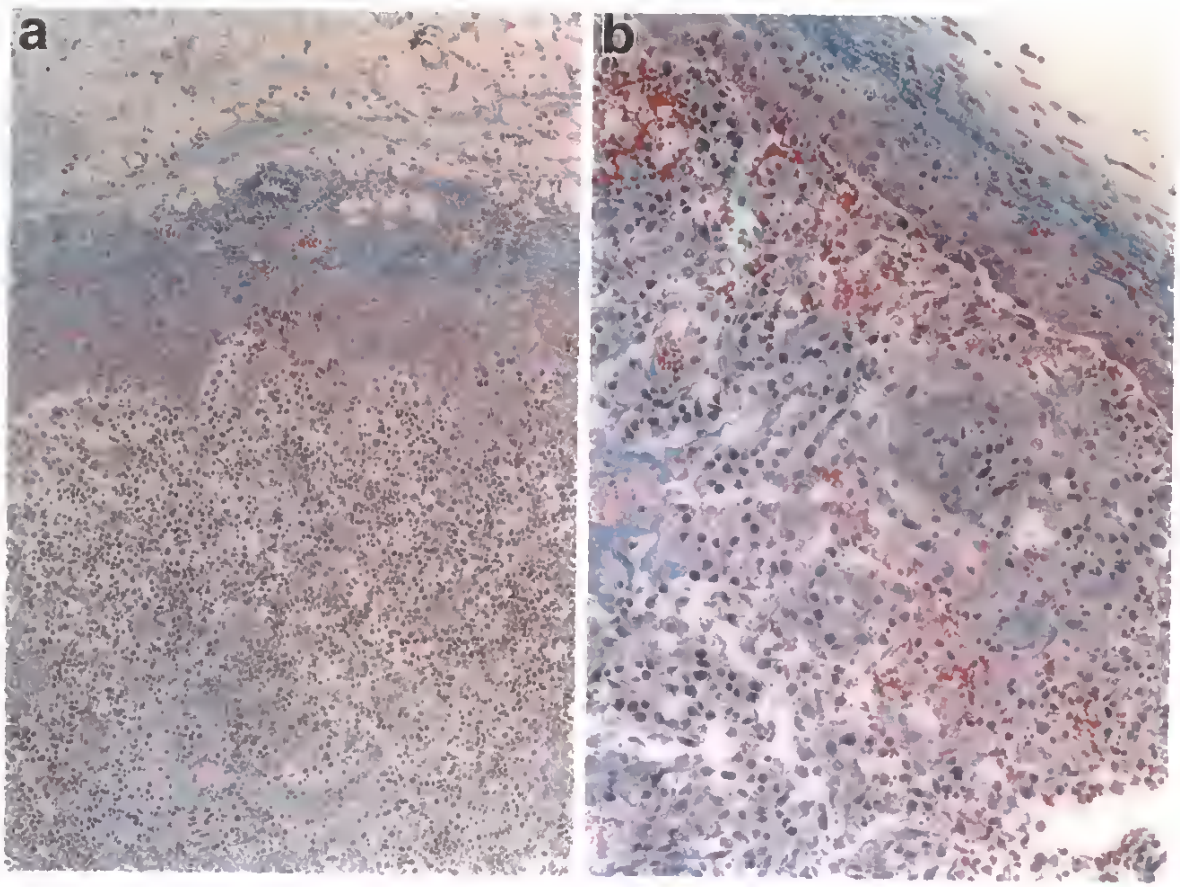


Figure 4-4. Standard H&E staining of 4-μ sections from KS tumors. Tumors were from mice that had received a 100 mg/kg dose of CA4DP 24 hr prior to assessment. Magnification was ×10 (a) and ×40 (b).

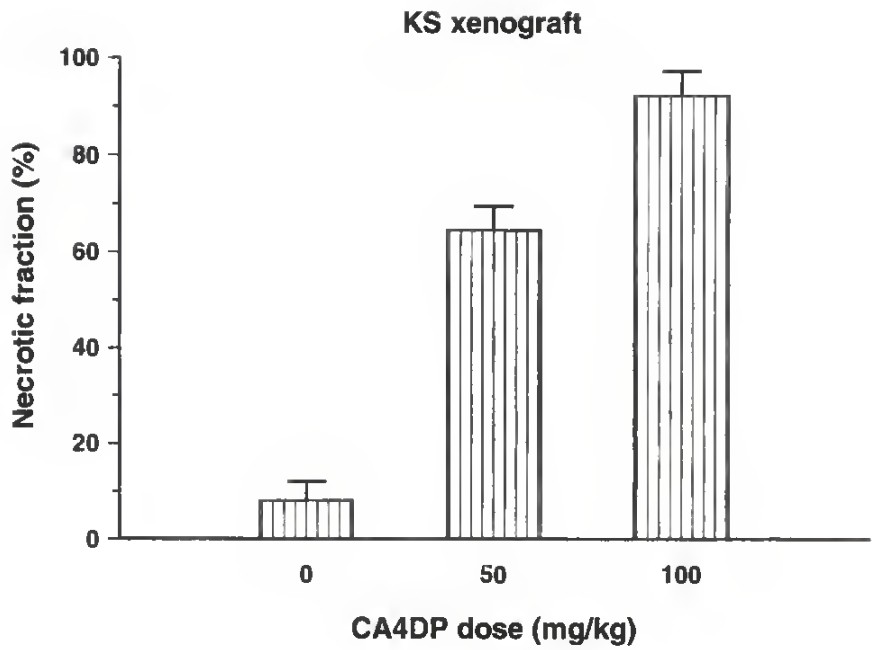


Figure 4-5. The extent of necrosis in KS xenografts assessed 24 hr after the administration of single doses (50 or 100 mg/kg) of CA4DP. Data are the mean ± SE.

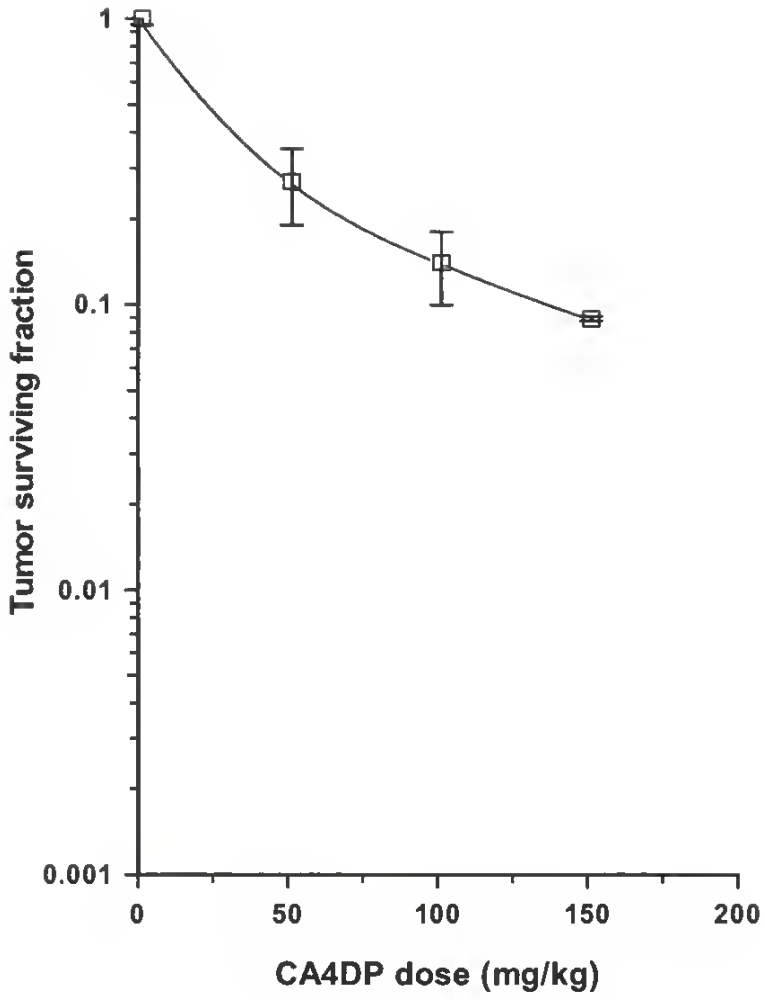


Figure 4-6. Tumor cell killing in KS tumors treated with increasing doses of CA4DP. Data were determined 24 hr after drug treatment and are the mean ± SE of at least 6 tumors.

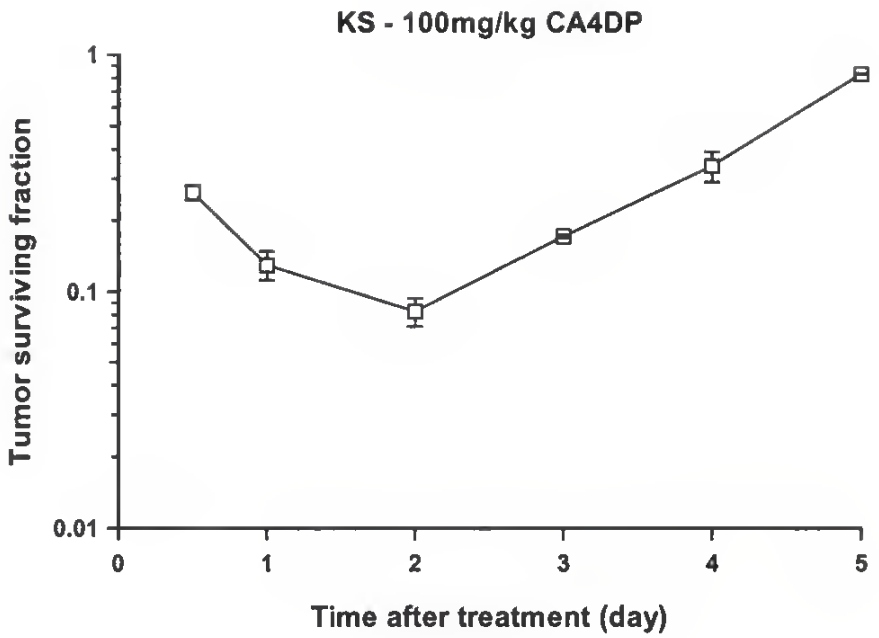


Figure 4-7. Tumor cell killing in KS tumors treated with a 100 mg/kg CA4DP. Clonogenic cell survival assay was performed at a specified time after drug treatment. Data are the mean ± SE of at least 6 tumors.

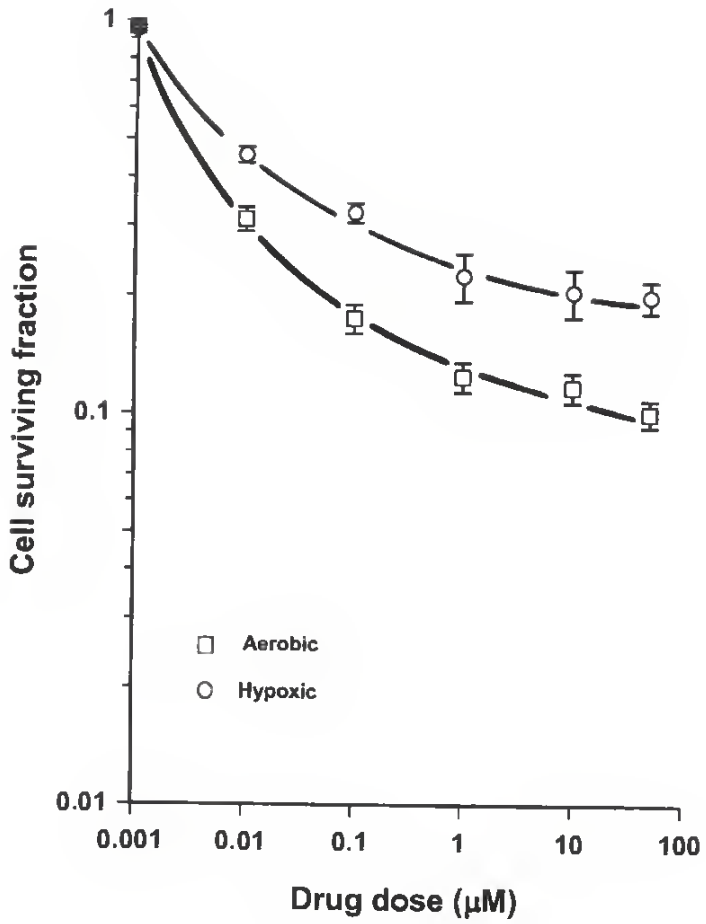


Figure 4-8. The cytotoxicity profile of CA4DP against KSY-1 cells under hypoxic or aerobic conditions. The cells were exposed to CA4DP for 24 hr and the cell killing effects were assessed using clonogenic cell survival assay. Data are the mean ± SE of three experiments.

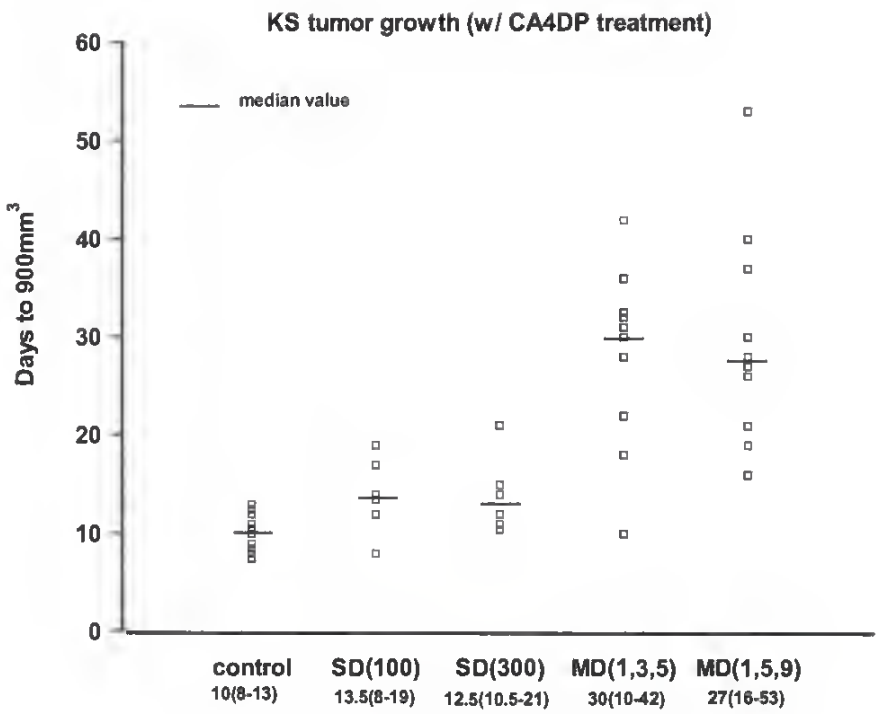


Figure 4-9. Growth delays in KS tumors with single or multiple doses of CA4DP treatment. Single dose (SD) of CA4DP (100 or 300 mg/kg) was administered on day 1. Multiple doses (MD) of CA4DP (100 mg/kg) were administered on days 1, 3, and 5 or on days 1, 5, and 9. Each datum point represents an individual animal.

Table 4-1. GROWTH DELAY IN KS XENOGRAFTS CAUSED BY SINGLE OR MULTIPLE DOSES OF CA4DP INJECTION IN TUMOR-BEARING MICE

GROUP	MEDIAN TIME TO 900mm^3 (DAYS)	GROWTH DELAY (DAYS)	SIGNIFICANCE[3] VS CONTROL	SIGNIFICANCE[3]
1. Control	10.0			
2. S.D.(100)[1]	13.5	3.5	NS	4 vs 2, 5 vs 2 $P< 0.025$
3. S.D.(300)[1]	12.5	2.5	NS	4 vs 3, 5 vs 3 $P< 0.025$
4. M.D. (1,3,5)[2]	30.0	20.0	$P< 0.025$	
5. M.D. (1,5,9)[2]	27.0	17.0	$P< 0.025$	

[1] Single dose of 100 or 300 mg/kg CA4DP were used in the treatment.
[2] 100 mg/kg CA4DP was administered to tumor-bearing mice on days 1, 3, and 5 or on days 1, 5, and 9.
[3] Determined by Wilcoxon Rank-Sum Test while compared the probability distributions of growth times of control versus treated tumor (α= .025).

CHAPTER 5
STUDY OF THE EFFICACY OF CA4DP IN COMBINATION WITH CONVENTIONAL ANTI-CANCER THERAPIES IN KS XENOGRAFTS

Introduction

AIDS-related KS is characterized by a heterogeneous presentation and an aggressive clinical course. It often presents as multiple, symmetric, cutaneous lesions. Because of the heterogeneous presentation of AIDS-KS, no single treatment regimen can be recommended for all patients. Therapy for AIDS-KS is not curative and, to date, no therapy has been unequivocally proven to impact survival, with the possible exception of interferon-α (Morris and Valley, 1996). The extent and rate of progression of AIDS-KS and the severity of the underlying HIV infection are factors used to determine the best treatment approach for individual patients.

Since cell proliferation and angiogenesis are the two key mechanisms involved in KS tumor growth, most of the treatments target one or both of them. Antiproliferative agents commonly used are chemotherapy, interferon and radiotherapy. AIDS-KS lesions are exquisitely sensitive to radiation therapy (Hill, 1987). Symptoms caused by mass effects (pain and lymphadenopathy) are best treated with radiation therapy because a response can be more rapidly achieved. Significant responses are reported in essentially all lesion treated with radiation therapy, including pulmonary KS lesions refractory to conventional chemotherapy

regimens (Hill, 1987). Complications such as mucositis and ulceration of skin and tissue may result after radiation therapy.

Both single-agent and combination chemotherapy have been used in patients with various stages of AIDS-KS. Single-agent chemotherapy produces responses in ~25% of patients. The duration of response reported has ranged from 1 to 9 months, and all patients relapse eventually after discontinuing therapy (Morris and Valley, 1996). Several combinations of chemotherapeutic agents have been investigated in an attempt to increase efficacy and to diminish toxicity by using lower dosages of individual agents. For example, since vincristine and vinblastine have exhibited significant activity in AIDS-KS, they were combined in a weekly alternating schedule in an attempt to reduce toxicity (Sung *et al.*, 1997). Still, because patients with HIV infection tend to be very susceptible to chemotherapy-induced toxicity, combination chemotherapy is reserved for treatment in patients with rapidly progressive or potentially life-threatening disease.

The optimal therapy for AIDS-KS has not yet been determined. Additional efforts in the management of AIDS-KS are directed at the underlying pathogenesis of the disease. Several antiangiogenic agents are being evaluated, including the heparin analog pentosan polysulfate, recombinant platelet factor-4, fumagillol derivatives, bacterial cell wall complexes, and suramin (Morris and Valley, 1996). Preclinical studies also examined the effects of antisense oligonucleotides which target bFGF and VEGF mRNA on KS growth. It was shown that the antisense oligonucleotides could block angiogenensis and KS lesion formation in nude mice (Ensoli *et al.*, 1994).

Because KS is a highly vasoactive neoplasm, directly targeting the actively growing vessels of the tumor may be suitable for KS treatment. Previous studies in our laboratory have investigated the efficacy of the vascular targeting agent CA4DP in the model of AIDS-KS. Results have shown that 100 mg/kg dose of CA4DP caused ~90% tumor necrosis in KS xenografts 24 hr after treatment (Figure 4-5), and that repeated doses, not single dose, of CA4DP treatment caused significant growth delay in KS xenografts (Figure 4-9). Previous studies also indicated that CA4DP alone were unable to eliminate the tumor completely, and a small, nevertheless viable, rim of tumor remained. These remaining viable tumor cells continue to proliferate, which may explain the lack of change in tumor growth following a single dose of CA4DP treatment. Therefore, the application of antivascular agents will need to be given in conjunction with conventional anticancer therapies. Our studies have shown that CA4DP can significantly enhance tumor response to radiation in KHT sarcoma (Li *et al.*, 1998), and others also demonstrated an effective enhancement of antitumor effects of cisplatin and 5-FU by combining with CA4DP (Chaplin *et al.*, 1999; Grosios *et al.*, 2000). Radiation and chemotherapy are the standard therapies for patients with various stages of AIDS-KS. In the present investigations we have examined the efficacy of combining CA4DP with ionizing radiation or chemotherapeutic agents in the model of Kaposi's Sarcoma.

Before examining the effects of CA4DP in combination with chemotherapeutic agents in KS xenografts, we screened the sensitivity of KSY-1 cells to several agents clinically used to treat KS patients, aiming to find the

suitable agents for the combination study *in vivo*. These agents are cisplatin, vinblastine, doxorubicin, and VP-16.

Materials and Methods

Cell Culture and Drug Sensitivity Study

KSY-1 cells (ATCC, Rockville, MD) were cultured in positively charged $Cell^{+}$ tissue culture flasks from SARSTEDT (Newton, NC). The cells were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY) with 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS, and were passed weekly.

To test the sensitivity of KSY-1 cells to chemotherapeutic agents cisplatin, vinblastine, doxorubicin, and VP-16, KSY-1 cells were plated in 60 mm petri dishes at 1×10^{5} cells/dish. On day 3, cisplatin (Bristol-Myers Squibb Co., Princeton, NJ), vinblastine (Fujisawa USA, Inc., Deerfield, IL), doxorubicin (Gensia Laboratories, Ltd., Irvine, CA), and VP-16 (Bristol-Myers Squibb Co., Princeton, NJ) injection solutions were diluted in 0.9% sterile saline before drug treatment. KSY-1 cells were then treated with these agents at specified concentrations for 1 hr. The dishes were then washed with PBS and replenished with fresh RPMI 1640 medium. 23 hr later, cells in each group were trypsinized, counted, and plated into 60 mm petri dishes for clonogenic cell survival assay. After 2 weeks of incubation at 37°C, colonies of 50 or more cells were counted with the aid of a dissecting microscope. Cell surviving fractions were calculated as the ratio of colonies counted in treated versus untreated group.

KS Xenografts and Treatments

KS xenografts were initiated by injecting the flanks of 6-8-week-old athymic NCR nu/nu mice (Frederick Laboratories, Frederick, MD) with 1×10^6 KSY-1 cells and were serially passed by subcutaneous transplantation of tumor pieces. Macroscopic tumors were available for experiments 3-4 weeks later. Tumor-bearing mice were allocated to groups and receive either no treatment or different treatment strategies. CA4DP, cisplatin, and vinblastine all were injected intraperitoneally in a volume of 0.01 ml/g animal body weight. In the combination experiments, cisplatin or vinblastine was administered 1 hr before CA4DP injection. For radiation treatment, tumors were irradiated in unanesthetized mice using a ^{137}Cs source operating at a dose rate of 1.5 Gy/min. In the combination studies, CA4DP was given 0.5-1 hr after radiation treatment.

Measurement of Tumor Response

Clonogenic cell survival in treated or untreated tumors was assessed using an *in vivo* to *in vitro* clonogenic cell survival assay as previously described (Allalunis-Turner and Siemann, 1986; Siemann, 1995). Briefly, 24 hr after treatment, tumor-bearing mice were killed and their tumors excised and then dissociated to a single cell suspension using an enzyme cocktail (0.025% collagenase, 0.05% pronase, and 0.04% DNase). The cells then were mixed with 10^4 lethally irradiated cells in α-MEM medium containing 10% fetal bovine serum and plated into 60 mm petri dishes. After 2 weeks of incubation at 37°C, colonies of 50 or more cells were counted with the aid of a dissecting microscope. Tumor surviving fractions were determined by multiplying the calculated fraction

of surviving cells by the ratio of cells recovered in treated versus untreated tumors.

Tumor Growth Delay Assay

Once KS xenografts had reached a minimum size of 200 mm^3, the mice were allocated to different groups for CA4DP and cisplatin treatment. A 100 mg/kg dose of CA4DP given on days 1, 3, and 5 or a single dose of 10 mg/kg cisplatin was used in the single-agent treatment studies. For the combination treatment group, a 10 mg/kg dose of cisplatin was administered on day one, followed by repeated injection of 100 mg/kg CA4DP on days 1, 3, and 5. After treatment, tumors were measured daily using calipers and the perpendicular diameters were determined. Tumor volumes were estimated using the equation: Volume = $4\pi r^3/3$, $r = (a+b)/4$, where a and b are the perpendicular diameters. The time for each tumor to reach a size of 900 mm^3 was recorded.

Results

Figure 5-1 illustrates the killing effect of various doses of radiation in KS xenografts either alone or with a 100 mg/kg dose of CA4DP given 0.5-1 hr after radiation. The results showed that when KS-bearing mice were treated with a combination of radiation and CA4DP, the extent of tumor cell killing was increased significantly, compared with that seen for radiation alone. The addition of CA4DP to the treatment protocol reduced tumor cell survival 10-100-fold below that for radiation alone. Furthermore, the combination treatment appeared to alter the shape of the tumor cell survival curve compared to that for radiation

alone, which suggests that the addition of CA4DP to the treatment influenced the radiation-resistant hypoxic cell subpopulation in KS tumors.

Since CA4DP alone is unable to eliminate the tumor completely, the possibility of producing even greater anti-tumor effects by adding a second agent to specifically target the rim of viable tumor cells surviving CA4DP treatment was investigated. Because KS xenografts were initiated by injecting KSY-1 cells in nude mice, our initial studies tested the sensitivity of KSY-1 cells to several agents that are clinically used to treat KS patients. Results demonstrated that cisplatin, vinblastine, doxorubicin, and VP-16 all showed dose-dependent killing effects in KSY-1 cells (Figure 5-2). However, different dose profiles were observed in different treatment groups. Vinblastine showed toxicity to KSY-1 at the lowest dose range compared to the other agents (Figure 5-2b vs Figures 5-2a, 5-2c, 5-2d). Cisplatin showed the strongest tumor cell killing effect at high concentrations. Because vinblastine and cisplatin are commonly used agents in KS patients, and because of the good response that KSY-1 cells showed to these two agents, vinblastine and cisplatin were chosen as the agents to be used in the combination studies with CA4DP *in vivo*.

The results of treating KS-bearing mice with a range of doses of cisplatin or vinblastine either alone or in combination with 100 mg/kg CA4DP are illustrated in Figures 5-3 and 5-4. In these studies CA4DP was administered 1 hr post-chemotherapy and clonogenic cell survival was assessed 23 hr later. The results demonstrated that when KS-bearing mice were treated with a combination of cisplatin (or vinblastine) and CA4DP, the extent of tumor cell killing could be

increased significantly, compared to that seen for either agent alone (Figures 5-3 and 5-4). The addition of CA4DP to the treatment reduced tumor cell survival at least 10 folds below that for cisplatin or vinblastine treatment alone.

In subsequent studies the combination of cisplatin plus CA4DP was evaluated in KS-bearing mice using tumor growth delay as the response endpoint. Single dose treatment with cisplatin alone (10 mg/kg) resulted in significant growth delay in this tumor model (Figure 5-5, Table 5-1). This response was further enhanced by combining cisplatin with CA4DP. Indeed tumor-bearing mice treated with this combination showed significantly greater growth delay than that seen for either cisplatin or CA4DP treatment alone (Figure 5-5, Table 5-1). It was also observed that CA4DP treatment alone did not cause significant weight loss in the treated mice, and it did not increase the toxic effects of cisplatin in the combination treatment compared to cisplatin treatment alone (Table 5-1).

Discussion

Kaposi's Sarcoma is the most common malignancy diagnosed in AIDS, occurring in approximately 20% of these patients. Several chemotherapeutic agents including vinca alkaloids, etoposide, doxorubicin, and cisplatin are used to treat AIDS-KS patients. Opportunistic infections occur in 30-50% of AIDS-KS patients receiving cytotoxic therapy (Schwartsmann *et al.*, 1998). In addition, drug-related toxicities are observed in almost all patients. In spite of these limitations, cytotoxic agents are usually an important component of the therapeutic armament for severe forms of symptomatic AIDS-KS.

The management of AIDS-KS still presents a challenge to clinicians and scientists. The development of novel therapeutic agents to improve outcomes in patients is badly needed. Because KS is a highly vasoactive neoplasm with a cellular origin suggested to be endothelial cell derived (Kroll and Shandera, 1998), we have investigated the efficacy of CA4DP in an *in situ* model of this disease. Results from previous studies were encouraging. Single dose of CA4DP treatment resulted in a rapid induction of vascular damage in the tumors such that 4 hr after treatment there existed an almost complete vascular shutdown (Figure 4-1). This was followed by haemorrhagic necrosis and extensive tumor cell killing in KS xenografts. Still, despite the promising results achieved, cure with an individual modality (monotherapy) is often difficult and the application of vascular targeting approach will undoubtedly be investigated in a neoadjuvant setting. Since CA4DP itself is unlikely to eradicate all of the tumor, in the present studies we have examined the effects of combining CA4DP with radiation and chemotherapeutic agents in KS xenografts.

Initial studies focused on the efficacy of combining CA4DP with radiation therapy. Previous studies showed that the vascular shutdown and subsequent induction of necrosis after CA4DP treatment is not complete, thus leaving areas of viable tumor cells from which the tumor could regrow (Li *et al.*, 1998; Figure 4-4). Interestingly, cells surviving treatment with CA4DP tend to be located in areas at the tumor periphery near normal tissues, most likely those areas supplied by normal tissue vessels (Figure 4-4). This residual tissue is likely to be well-oxygenated and hence responsive to radiation. Results from the clonogenic cell

survival investigations demonstrated that combining CA4DP with radiation treatment could significantly enhance the tumor cell killing effect compared to radiation alone. It is well established that the aberrant vascular morphology, spatial heterogeneity in vessels, and metabolic microenvironment associated with solid tumors, can have significant adverse effects on the efficacy of radiation therapy. Treatment with CA4DP eliminates many of these problem areas by causing extensive hemorrhagic necrosis in the centers of tumors (Li *et al.*, 1998; Horsman *et al.*, 1998). Results showed that when CA4DP was used in conjunction with radiotherapy, the tumor's hypoxic cell population appeared to be significantly impacted. For radiation treatment alone, the "break" in the KS cell survival curve reflects the presence of the hypoxic cell population in this tumor (Figure 5-1). It was observed that the inclusion of CA4DP in the treatment strategy altered the radiation dose response curve: the "break " seen with radiation treatment alone was eliminated with the combination treatment. It demonstrates that CA4DP may improve the radiation response of tumors by impacting the radiation refractory hypoxic cell subpopulation of tumors. Such a conclusion is consistent with previous histological evaluations in KS xenografts. 100 mg/kg CA4DP could induce massive central necrosis in KS tumors leaving viable cells only at the periphery (Figure 4-4). Taken together, these findings suggest that CA4DP and radiotherapy are acting in a complimentary fashion at the microregional level, i.e. the vascular targeting agent is preferentially eliminating the poorly oxygenated and hence radioresistant tumor cell subpopulations.

The present studies also investigated the efficacy of combining CA4DP with conventional chemotherapeutic agents. The rationale for such combinations was based on two factors. First, it is logical to combine drugs or treatment modalities with different mechanisms of action or different dose-limiting toxicities to obtain a therapeutic benefit. Second, as discussed above, our CA4DP studies showed that this agent, though effective at inducing large scale tumor necrosis, failed to eliminate a viable rim of tumor cells surviving at the tumor's periphery (Figure 4-4). Consequently, CA4DP treatment was combined with cisplatin or vinblastine therapy. Due to the rapid action of CA4DP against the tumor vasculature (Figure 4-2), in the combination studies, cisplatin and vinblastine treatment preceded CA4DP so as not to interfere with the tumor uptake of the chemotherapeutic agents. The results showed that the CA4DP-chemotherapy combination led to significantly enhanced tumor cell killing (Figures 5-3 and 5-4). Most critically this enhancement could still be achieved at the maximum tolerated single doses of cisplatin (20 mg/kg) and vinblastine (15 mg/kg). Clinically, the application of both vinblastine and cisplatin are limited by their toxic side effects, especially neurotoxicities. Indeed, the long terminal elimination half life of vinblastine associated with its low elimination constant may explain its low MTD in patients (Zhou and Rahmani, 1992). In contrast, the antivascular effects of CA4DP were achieved at doses less than 1/10 of the MTD and without detectable morbidity. In the present studies, it is clearly seen that CA4DP potentiates the anticancer effects of cisplatin and vinblastine significantly at very low doses. Therefore,

alternatively, the same anti-tumor effects could be achieved at far lower doses of cisplatin and vinblastine when CA4DP was included in the treatment.

From previous studies we know that multiple doses of CA4DP were far more effective at inhibiting KS growth than single dose treatments. Still, because of the viable rim of tumor cells left after CA4DP treatment alone, we reasoned that administering a second agent along with CA4DP might prove to be a much more efficient application of this agent. Combining cisplatin with CA4DP proved to be more effective at inhibiting KS growth than either treatment alone (Figure 5-5, Table 5-1). The results showed that the combination of cisplatin and CA4DP led to a far superior growth delay and an additive anti-tumor effect in KS xenografts. This growth delay caused by the combination treatment was achieved with no more toxicity detected beyond that seen with cisplatin treatment alone. Taken together, these data demonstrated that combining the antivascular agent CA4DP with another agent which kills the tumors cells directly is a feasible and effective way of inhibiting KS growth.

In conclusion, the vascular targeting agent CA4DP could significantly enhance the antitumor effects of radiation therapy and chemotherapeutic agents. These findings from the present studies illustrate that potential utility of employing a treatment strategy that combines a vascular targeting approach with radiation or chemotherapy to elicit increased antitumor efficacy in KS. Phase I clinical trials with CA4DP are nearly complete. The present results suggest that the evaluation of CA4DP in patients with AIDS-KS be considered in future Phase II studies.

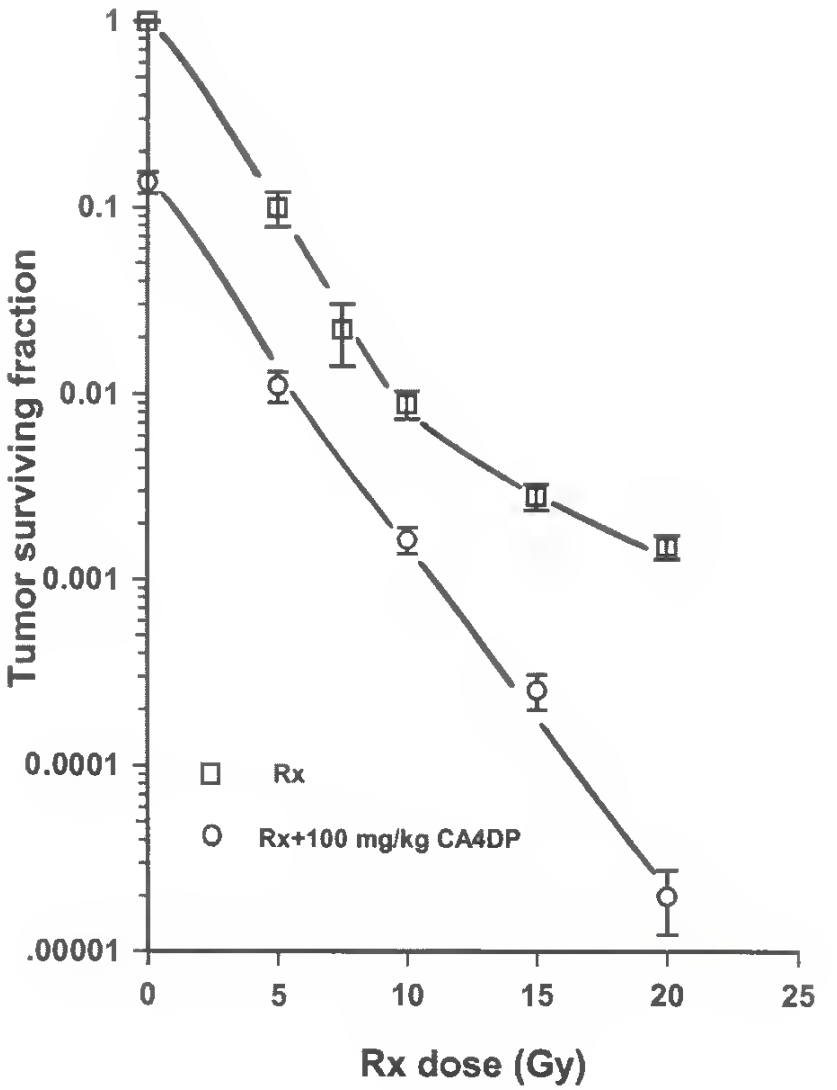
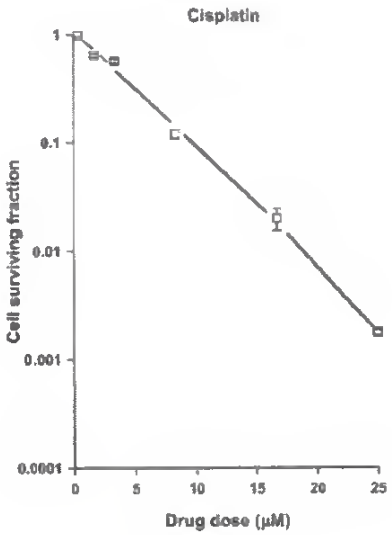
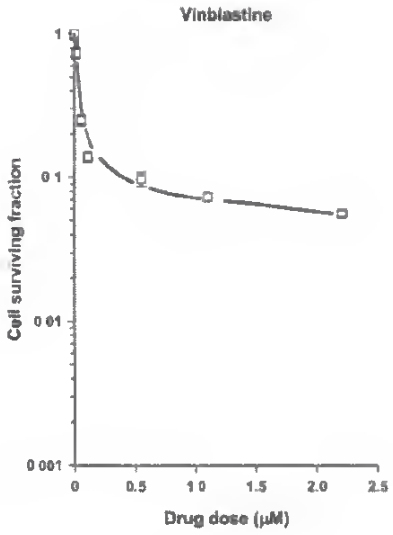


Figure 5-1. Tumor cell survival in KS xenografts treated with a 100 mg/kg dose of CA4DP 1 hr after a range of doses of radiation. Results are the mean ± SE of 3 experiments.

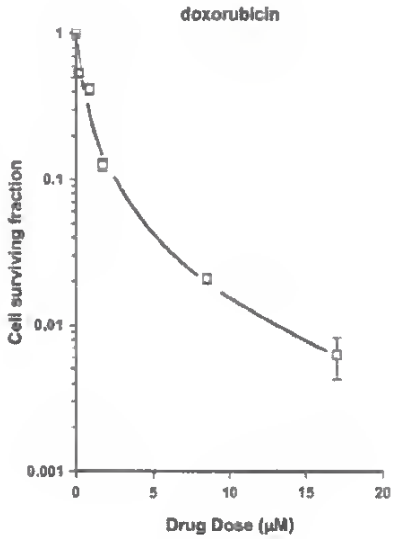
a.



b.



c.



d.

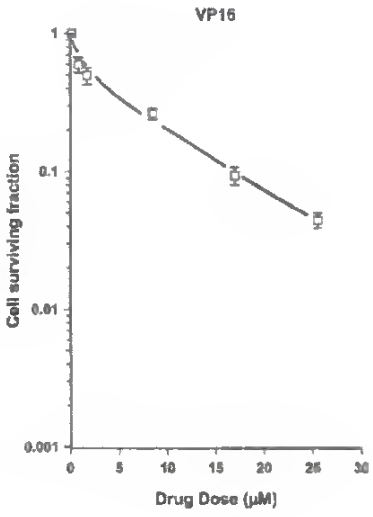


Figure 5-2. KSY-1 cell survival after 1-hr treatments with chemotherapeutic agents. Clonogenic cell survival assay was performed 23 hr after drug treatment. Data are the mean ± SE of three experiments. a) Cisplatin; b) Vinblastine; c) Doxorubicin; d) VP-16.

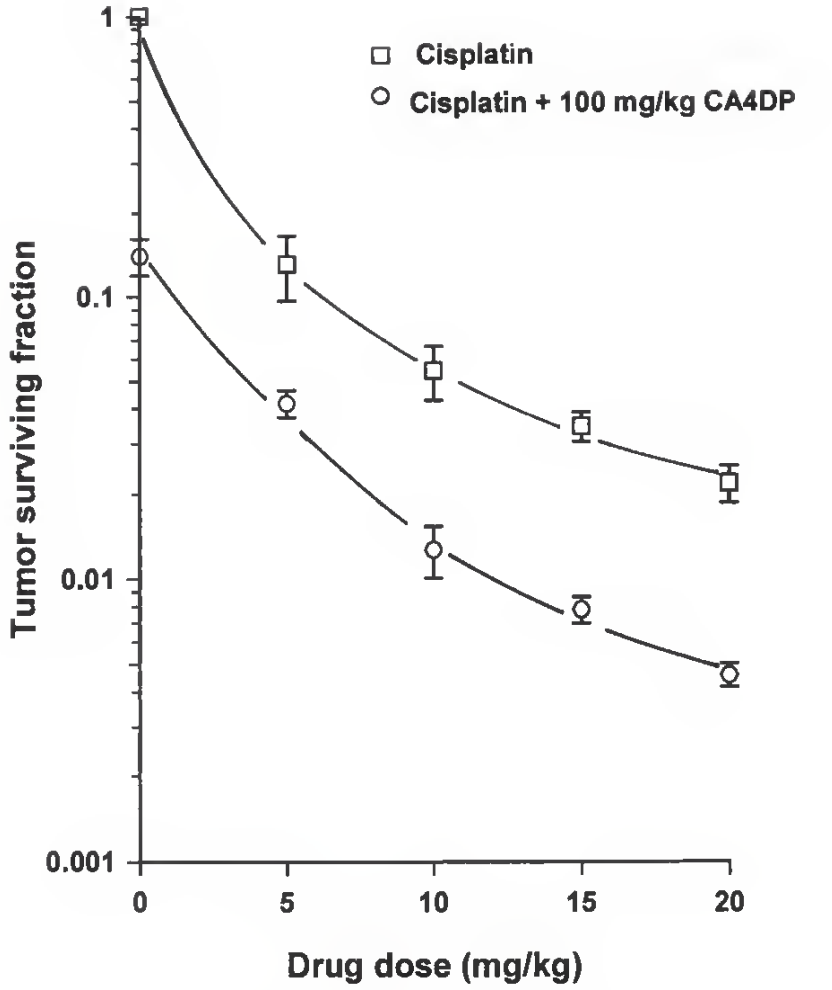


Figure 5-3. Tumor cell survival in KS xenografts treated with a 100 mg/kg CA4DP 30 min after a range of doses of cisplatin treatment. Data are the mean ± SE of at least 6 tumors.

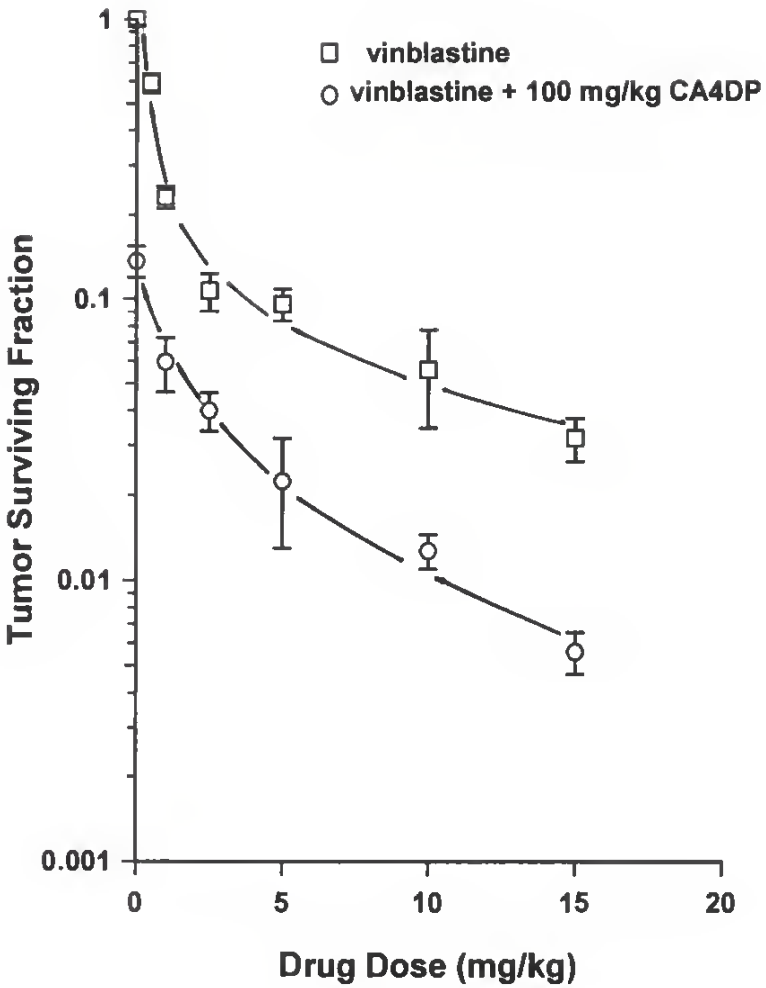


Figure 5-4. Tumor cell survival in KS xenografts treated with a 100 mg/kg CA4DP 30 min after a range of doses of vinblastine treatment. Data are the mean ± SE of at least 6 tumors.

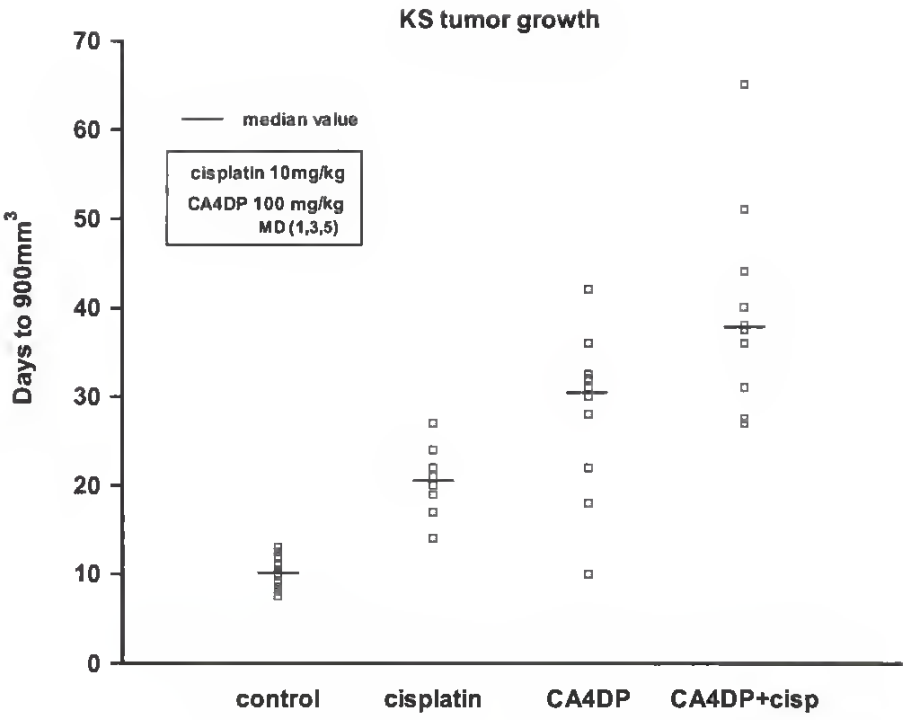


Figure 5-5. Growth delays in KS tumors with cisplatin, CA4DP, and CA4DP + cisplatin treatment. 10 mg/kg cisplatin was administered on day 1 for cisplatin treatment. 100 mg/kg CA4DP was given on days 1, 3, and 5 for CA4DP treatment. For the combination treatment. 10 mg/kg cisplatin was given on day 1, followed by three 100 mg/kg dose fractions of CA4DP on days 1, 3, and 5. Each data point represents an individual animal.

Table 5-1. GROWTH DELAY IN KS XENOGRAFTS CAUSED BY CISPLATIN AND CA4DP TREATMENT

GROUP	MEDIAN TIME TO 900mm^3 (DAYS)	GROWTH DELAY (DAYS)	SIGNIFICANCE[4] VS GROUP 1	SIGNIFICANCE	>10% WEIGHT LOSS
1. Control	10.0	--	--		0/10
2. Cisplatin[1]	20.5	10.5	P< 0.025	4 vs 2 P< 0.025	2/8
3. CA4DP(1,3,5)[2]	30.0	20.0	P< 0.025	4 vs 3 P< 0.025	0/10
4. CA4DP + cisp[3]	38.0	28.0	P< 0.025		2/10

[1] Single dose of 10 mg/kg cisplatin given on day 1 (tumor size 200 mm^3).
[2] 100 mg/kg CA4DP administered on days 1, 3, and 5.
[3] 10 mg/kg cisplatin given on day 1, followed by 100 mg/kg CA4DP on days 1, 3, and 5.
[4] Determined by Wilcoxon Rank-Sum Test while compared the probability distributions of growth times of control versus treated tumor (α= .025).

CHAPTER 6
SUMMARY AND PERSPECTIVE

The vascular targeting approach has received considerable attention in recent years. This type of therapy takes advantage of the uniqueness of the endothelium of established tumor capillaries and their supporting structures. The use of antivascular agents offers promise for the treatment of solid tumors as the growth of a tumor above a volume of 1 mm^3 inevitably demands vascularization to supply nutrients for proliferating tumor cells (Folkman, 1986). The current challenge is to develop treatments that are highly selective for the tumor vasculature, thus enabling systemic administration in well-tolerated regimes. The tubulin binding agent CA4DP may provide the lead for developing such selective vascular targeting drugs, based on its promising antivascular effects in preclinical models (Dark *et al.*, 1997; Li *et al.*, 1998; Horsman *et al.*, 1998) and encouraging results from Phase I clinical trials (Randal, 2000). My studies during the last several years have focused on gaining insight into the mechanisms underlying the antivascular action of CA4DP and examining the antitumor efficacy of CA4DP in a xenograft model of Kaposi's Sarcoma.

The present studies demonstrated that CA4DP not only inhibited tubulin polymerization, endothelial cell migration and attachment, but also induced endothelial cell apoptosis. An important finding from our studies was that CA4DP

had selective toxicity against proliferating endothelial cells. By comparing the responses of tumor, endothelial, and fibroblast cells to CA4DP, we can clearly see that proliferating endothelial cells are significantly more sensitive to CA4DP treatment. Other *in vitro* studies indicated that CA4DP had the ability to interfere with endothelial cell behavior and this might be a way it exerts its action. Although further studies are needed to fully appreciate why endothelial cells are more sensitive to CA4DP treatment than other types of cells, our findings provide a basis for the *in vivo* efficacy of CA4DP.

Two factors may possibly contribute to the high sensitivity of endothelial cells to CA4DP. First, endothelial cells may contain higher level of alkaline phosphatase than other cell types, resulting in an increased efficacy of CA4DP in endothelial cells. It has been observed that compared to fibroblasts, endothelial cells showed higher alkaline phosphatase activity, located on the cell membrane (Gallo *et al.*, 1997). Second, endothelial cells may be more sensitive to their cell shape change than other cells. Therefore, with CA4DP treatment, they could more easily detach and undergo apoptosis. Recent studies in our laboratory have focused on both these issues. Currently we are attempting to measure the activity of alkaline phosphatase in different cell types. A study of the relationship between phosphatase activity and responsiveness to CA4DP in a range of cell types would provide further insight into the mechanism of action of this drug. In addition, we have initiated investigations using deconvolution microscopy to observe endothelial cell shape changes after CA4DP treatment on a 3-dimentional level.

These studies will help to further elucidate the mechanisms responsible for the endothelial cell specific effects of CA4DP.

The present studies also demonstrated significant antivascular effect of CA4DP in KS xenografts. CA4DP caused rapid vascular shutdown which resulted in extensive haemorrhagic necrosis in these tumors. Multiple doses of CA4DP treatment alone already caused long and significant growth delays in KS tumors. Most importantly, CA4DP caused these effects at doses less than 1/10 of the MTD, making CA4DP a promising vascular targeting agent with a wide therapeutic window.

The mechanisms of action of CA4DP are likely related to its tubulin-binding properties which may be responsible for the rapid endothelial cell damage, neovascular shutdown and subsequent haemorrhagic necrosis. Drugs that work by this mechanism are unlikely to eradicate the tumor as a single agent but may enhance the efficacy of other anticancer treatments. That this may indeed be the case was supported by our *in vivo* investigations which demonstrated that enhanced tumor effects could be achieved by combining CA4DP with standard anticancer therapies, such as radiation and chemotherapy. Most critically these enhancements were still achieved at the maximum tolerated doses of cisplatin and vinblastine.

Both our *in vitro* and *in vivo* studies with CA4DP have demonstrated its strong antivascular effect, which is a common feature of tubulin-binding agents. However, CA4DP appears to be a much better vascular targeting agent compared to some other tubulin-binding agents such as colchicine and vinblastine. Recently,

a novel combretastatin A-4 derivative AC7700 (Hori *et al.*, 1999) and another agent which has similar structure to combretastatin A-4, ZD6126 (being developed by AstraZaneca Pharmaceuticals, Wilmington, DE), have also been shown to have strong antitumor effects due to their rapid shutdown of the tumor blood flow. This made us believe that the similar antivascular properties of CA4DP and the other two agents are closely related to their structural specificity. Moreover, another feature that makes CA4DP unique is its short half-life (30-45 min in nude mice) *in vivo* compared to that of vinblastine (24 hr) and colchicine (20 hr). The very short half-life of CA4DP allows this agent to act primarily on the vascular endothelial cells, making CA4DP a promising antivascular agent with very low level of toxicity seen in preclinical studies.

CA4DP treatment causes massive necrosis in KS tumors, which can induce inflammatory response in cancer patients. Local inflammatory response is helpful in getting rid of the necrotic cell debris in the tumor and also in killing some other survived tumor cells. However, when the response mediators, for example, lymphokines and antibodies go into the blood stream, they could cause toxicities due to the non-specific inflammatory response. Therefore, anti-inflammatory agents such as corticosteroid hormones should be used along with CA4DP treatment if necessary.

Treatment for AIDS-KS continues to present a challenge for physicians. The dose-limiting toxicities of radiation and chemotherapy severely limit these therapies in AIDS-KS patients. The present studies demonstrate that the combination of CA4DP with conventional anticancer therapies may prove to be

an effective way to achieve a greater therapeutic benefit. The same antitumor effects could be gained at far lower doses of radiation and chemotherapeutic agents when CA4DP was included in the treatment. Alternatively, greater antitumor effectiveness was achieved at maximally tolerated conventional anticancer therapy when combined with CA4DP. Our findings therefore suggest a possible application for CA4DP in the clinical management of KS. Phase I clinical trials with CA4DP are nearly complete. The present results suggest that patients with AIDS-KS might benefit from CA4DP combination studies in future Phase II studies.

There are still areas of interests that could be pursued in future preclinical studies. For example, our studies have shown an interesting interaction between CA4DP and single dose of radiation in the combination treatment. Future studies might focus on the effects of combining CA4DP with fractionated radiation therapy, since clinically radiation is normally given on a multiple-dose schedule to cancer patients. The combination of CA4DP and multiple doses of anticancer agents might also be examined based on the same reason. Moreover, the potential effect of CA4DP on tumor metastasis will be worthwhile to test in future preclinical investigations.

The work shown in this dissertation has been presented at 5 national and international meetings. A portion of the studies of the *in vivo* effects of CA4DP has been published in the International Journal of Radiation Oncology Biology and Physics (Li *et al.*, 1998). Another three manuscripts based on the studies from

Chapter 2 to Chapter 5 have been finished and recently submitted for publication to the International Journal of Cancer and British Journal of Cancer.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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